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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Kensil

Application No.: 09/369,941

Filed: August 6, 1999

For: COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS AND USES THEREOF

Confirmation No.: 7453

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Examiner: Wilson, Michael C.

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DECLARATION OF DR. CHARLOTTE KENSIL UNDER 37 C.F.R. §1.132

Commissioner for Patents
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Sir:

I, Dr. Charlotte Kensil, Ph.D., do declare and state that:

1. I presently hold the position of consultant at Antigenics Inc. Antigenics Inc. is the owner of the entire right, title and interest in, to and under the invention described and claimed in the above-identified patent application.

2. I received a Ph.D. from the University of California, San Diego in 1981. My academic and technical experience and honors, and a list of my publications, are set forth in my *curriculum vitae*, attached hereto as Appendix 1.

3. I am the sole inventor of the invention described and claimed in the above-identified U.S. Application No. 09/369,941 ("the '941 application"). I have read and understand the '941 application. I have also read the Office Action dated January 31, 2003, the Amendment Under 37 C.F.R. § 1.111 ("the Amendment") that was filed on July 31, 2003, and the Office Action dated October 27, 2003 ("the Office Action"), including the references cited by the Examiner as a basis for the rejection under 35 U.S.C. § 103(a). I understand that an issue relevant to the rejection under 35 U.S.C. § 103(a) for obviousness is whether the claims are

limited to (i) the immunostimulatory oligonucleotides containing at least one unmethylated CpG dinucleotide ("CpG oligonucleotides"), and (ii) the saponins derived from *Quillaja saponaria* possessing immune adjuvant activity ("QS saponins"), that together are reasonably expected to display synergy in immune adjuvant activity. An immune adjuvant is a compound which, when administered to an individual or tested *in vitro*, increases the immune response to an antigen in the individual or test system to which the antigen is administered (see specification of the '941 application at page 10, lines 10-12). In particular, I understand that the Examiner questions whether synergy in immune adjuvant activity is a general attribute of the genus of CpG oligonucleotides and the genus of QS saponins, contending that only certain CpG oligonucleotides and certain QS saponins have been shown to exhibit such synergy. I present this declaration to address this issue.

4. I will first discuss the evidence that leads to the conclusion that synergy in immune adjuvant activity, exhibited when CpG oligonucleotides are used in combination with QS saponins, is expected to be a general attribute of the genus of CpG oligonucleotides.

5. The '941 application presents data showing the unexpected synergism of CpG oligonucleotides and QS saponins. For example, Example 1 (page 20) and Figure 1 of the '941 application show the synergistic immune adjuvant activity exhibited by the combination of QS-21 (a QS saponin) and phosphorothioate oligonucleotide 1758 (a CpG oligonucleotide). Example 4 (pages 22-23) of the '941 application shows synergistic immune adjuvant activity exhibited by the combination of QS-21 and the phosphorothioate oligonucleotide 1826 (a CpG oligonucleotide). International Publication No. WO 00/62800 ("Friede", attached hereto as Exhibit 1) presents data showing that synergistic immune adjuvant activity is exhibited by (1) the combination of QS-21 and oligonucleotide 1826 (a CpG oligonucleotide) (see, *e.g.*, Example 1 on pages 23-25 of Friede); and (2) the combination of QS-21 and oligonucleotide 2006 (a CpG oligonucleotide) (see, *e.g.*, Example 2 on pages 25-27 of Friede).

6. CpG oligonucleotides are generally expected to act in the same manner with respect to immune adjuvant activity, since CpG oligonucleotides exert their activity through the same receptor and thus share the same mechanism of action. The cellular response to CpG oligonucleotides is mediated by a Toll-like receptor, TLR9 (Hemmi *et al.*, 2000, *Nature*

408:740-745, attached hereto as Exhibit 2, "Hemmi"). The current model for the mechanism through which CpG oligonucleotides act to elicit an immunostimulatory response is that TLR9 directly binds the immunostimulatory oligonucleotides containing CpG dinucleotides and becomes endocytosed to then allow activated TLR9 to bind MyD88, IRAK, and TRAF6, which then causes a signaling cascade culminating in transcription of gene products responsible for the immunostimulatory effects seen when CpG oligonucleotides are administered (see Figure 1 of Wagner, 2001, *Immunity* 14:499-502, attached hereto as Exhibit 3, "Wagner"). The genus of CpG oligonucleotides is expected in general to work through the same mechanism and thus to demonstrate the same activity. Many different CpG oligonucleotides are known in the art and shown to have immune adjuvant activity (see, *e.g.*, Weiner *et al.*, 1997, *PNAS*, 94:10833-10837, attached hereto as Exhibit 4, "Weiner"; and Chu *et al.*, 1997, J. Exp. Med. 186:1623-31, attached hereto as Exhibit 5, "Chu", both cited by the Examiner in connection with the 35 U.S.C. § 103(a) rejection; see also U.S. Patent No. 6,406,705 (attached hereto as Exhibit 6, "the '705 patent"), where a group of CpG oligonucleotides were shown to have immune adjuvant activity and to exhibit synergy in immune adjuvant activity when administered with alum (another adjuvant) (see Example 1 at column 37, lines 5-67, and Fig. 3 of the '705 patent)). In view of Exhibit 2 and Exhibit 3, it is reasonable to conclude that the immune adjuvant activity of CpG oligonucleotides known in the art, and of CpG oligonucleotides in general, is mediated via the same signal transduction mechanism. The evidence that I discussed above shows for three different CpG oligonucleotides the property of synergism with QS-21 in immune adjuvant activity. Since CpG oligonucleotides in general share the same mechanism of action, I conclude that CpG oligonucleotides in general are reasonably expected to exhibit the property of synergism with QS-21 in immune adjuvant activity, and as discussed in Paragraphs 7-11 below, synergism with QS saponins generally.

7. I will now discuss why QS saponins generally, *i.e.*, not just QS-21, are expected to exhibit synergy in immune adjuvant activity with CpG oligonucleotides. In particular, QS saponins share a common structure that gives rise to the common immune adjuvant function, and thus would be expected to function in the same manner with respect to the property of synergism with CpG oligonucleotides. For example, QS-17, QS-18, and QS-21 are saponins that are derived from *Quillaja saponaria*. All are structurally very similar. Kensil *et al.* (1993, "Novel

Adjuvants from *Quillaja saponaria* Molina" in AIDS Research Reviews Volume 3 edited by Koffet *et al.* New York; attached hereto as Exhibit 7, "Kensil I") shows the structures of QS-17, QS-18, and QS-21 in Figure 2, derived from comparison of monosaccharide composition and molecular weight. Compared to the large portion of the structure that is identical, any differences are minor.

8. All QS saponins whose structure is known to me share two structural features - a triterpene backbone and a 2,3, glucuronic acid carboxyl group (see the paragraph spanning pages 1403 and 1404 of Soltysik *et al.*, 1995, *Vaccine* 13:1404-10, attached hereto as Exhibit 8, "Soltysik"). Modification of the triterpene aldehyde of the backbone inactivated the ability of QS-21 to stimulate immune response as manifested by antibody production and cytotoxic T lymphocyte activity (page 1408, second column, first full paragraph of Soltysik (Exhibit 8)).

9. Furthermore, evidence demonstrates that all QS saponins (even those additional to QS -17, -18, and -21) have very similar structures. Mild alkaline hydrolysis of a crude extract of *quillaja* bark containing QS saponins generates only two major structures: desacylsaponin -1 and -2 (DS-1 and DS-2) (see Higuchi *et al*, Phytochemistry 26:229-235 (1987), attached hereto as Exhibit 9, "Higuchi"). DS-1 was shown to contain glucuronic acid, galactose, xylose, fucose, rhamnose, apiose, and quillaic acid, whereas DS-2 contained these same components plus glucose (see Higuchi). Thus, DS-1 and DS-2 have very similar structures, differing only in whether or not glucose is present.

10. Unlike the majority of saponins from species other than *Quillaja saponaria*, all *Quillaja saponaria* saponins are acylated. QS-17, QS-18 and QS-21 are acylated at identical positions and removal of this acyl group decreases immune adjuvant activity as reflected by total IgG response as compared to acylated counterparts (see page 2808, paragraph spanning the first and second columns of Liu *et al.*, 2002, *Vaccine* 20:2808-15, attached hereto as Exhibit 10, "Liu" (Liu shows that deacylated QS-21 was inactive in inducing IgG2a and cytotoxic T lymphocyte (CTL) responses); and Kensil *et al.*, Vaccine 1992:35-40, attached hereto as Exhibit 11, "Kensil II"). Thus, there are specific structural components (of QS saponins) that influence immune adjuvant activity.

11. Because of the structural similarity of the QS saponins and the correlation of structure with function, and the evidence that QS saponin QS-21 exhibits synergy in immune adjuvant activity with CpG oligonucleotides, I conclude that synergy in immune adjuvant activity is reasonably expected to be a general property of the genus of QS saponins.

12. In the Office Action, the Examiner presents several reasons why he was not persuaded that CpG oligonucleotides "are generally expected to act in the same manner, and thus share the characteristic of synergism with saponins from *Quillaja saponaria* ..." (see the Office Action, page 10, first full paragraph). I will now address the Examiner's stated reasons.

13. First, the Examiner alleges that Weiner teaches that "oligonucleotides 1758, 1643, and 1812 induced different humoral effects (Fig. 1), therefore, different immunostimulatory oligonucleotides induce different effects" (see the Office Action, page 10, first full paragraph). It is true that there are variations in the degree of adjuvanticity for the different CpG oligonucleotides (as shown in Fig. 1 of Weiner). However, each CpG oligonucleotide none-the-less exhibits an immunostimulatory effect. It is to be noted that oligonucleotide 1812, which shows little effect in Fig. 1, contains methyl cytosines instead of unmethylated cytosines (page 10834, 3rd paragraph of column 1 of Weiner), and thus is not a "CpG oligonucleotide" as defined above and recited in the claims of the present application. Thus, although one might expect a quantitative difference between different CpG oligonucleotides (*e.g.*, in the degree of synergism seen when used in combination with a saponin adjuvant), there is no indication that reasonably provides an expectation of a significant qualitative difference in the result achieved (*i.e.*, immune response) between the CpG oligonucleotides (*e.g.*, in the ability to have at least some degree of synergism when used in combination with a saponin adjuvant). In fact, to the contrary, as discussed above, since it can reasonably be concluded that CpG oligonucleotides share a mechanism of action due to their shared CpG motif, it is expected that they would all exhibit synergism in immune adjuvant activity when combined with a QS saponin.

14. Second, the Examiner alleges that Agrawal *et al.*, U.S. Patent No. 5,968,909 (attached hereto as Exhibit 12, "Agrawal") teaches that "some oligonucleotides having unmethylated CpG dinucleotides can be used to reduce the immune response to the oligonucleotide" (see the Office Action, page 10, first full paragraph). A similar statement is

made by the Examiner on page 12, second paragraph of the Office Action. The Examiner's statements are incorrect. Agrawal teaches how to modify certain phosphorothioate oligonucleotides so that they are less immunostimulatory than the unmodified phosphorothioate oligonucleotides. Such a modification would be useful when the oligonucleotides' immunostimulatory effects are unwanted, *e.g.*, in antisense technology. Agrawal does not teach the use of a CpG oligonucleotide to reduce an immune response to the same or another nucleic acid (or to an antigen). In fact, Agrawal supports the position that CpG oligonucleotides all share immunostimulatory activity (see column 1, line 6o, to column 2, line 3 of Agrawal).

15. Third, the Examiner alleges that it cannot be determined

> what applicants mean by oligonucleotides sharing "the characteristic of synergism with saponin" or how such a conclusion was made. Therefore, immunostimulatory oligonucleotides having at least one CpG dinucleotides are not expected to have the same function.

See Office Action, page 10, 1st full paragraph. The statement that CpG oligonucleotides "share the characteristic of synergism with saponins from *Quillaja saponaria*" (see the Amendment, page 25, 1st full paragraph) means that CpG oligonucleotides generally exhibit synergy in immune adjuvant activity when used in combination with QS saponins. This conclusion can be drawn because, as I have discussed in detail above, (1) evidence demonstrates that combinations of certain CpG oligonucleotides and certain QS saponins elicit synergistic immune adjuvant activity; and (2) CpG oligonucleotides exert their activity through the same receptor and thus share the same mechanism of action (see paragraph 6, *supra*).

16. In the Office Action, the Examiner also points out in support of his position that "the claims encompass any immune adjuvant activity and are not limited to increasing a cellular immune adjuvant activity" and that "the basis of this rejection is that both oligonucleotide 1643 and 1758 as well as QS-21 enhanced a humoral response." See the Office Action, page 11, lines 2-3. Certainly, the claims are not limited to adjuvants that increase only a cellular immune response. However, just because CpG oligonucleotides function through a Toll-like receptor in the cell membrane, this does not indicate that the result of signal transduction from that receptor would be exhibited only as a cellular immune response and not a humoral immune response. As taught in Wagner (Exhibit 3), CpG oligonucleotides influence the adaptive immune response by

activating innate immune cells such as macrophages and dendritic cells (DCs). See Wagner (Exhibit 3), page 500, column 1, lines 9-14. It is well-known in the art that macrophages and DCs play critical roles in activating T cells (which mediate cell-mediated immune responses) as well as B cells (which mediate humoral immune responses, *e.g.*, antibody production) by functioning as antigen presenting cells and/or by promoting cytokine release. Furthermore, the '941 application teaches that administering a combination of QS-21 and a CpG oligonucleotide produces synergistic production of cytotoxic T lymphocytes (CTLs) (see Example 1 and Example 2, pages 20-21 of the '941 application) and synergistic production of antibodies (see Example 3 and Example 4, pages 21-23 of the '941 application). Thus, a person skilled in the art would expect that the synergistic immune adjuvant effect exhibited by CpG oligonucleotides when used in combination with QS saponins would be exhibited as both a cell-mediated and humoral immune response.

17. The Examiner further states in support of his position that

> the fact that oligonucleotides containing unmethylated CpG dinucleotides are all processed by the TLR9 does not indicate that all bind to the same degree or that all are processed with equal efficiency and does not take into account the possibility of other structures that might improve processing of the oligonucleotides. A showing that all oligonucleotides having at least one CpG dinucleotide are processed by the same mechanism does not allow one to conclude that all such oligonucleotides have the same activity. Some are more immunogenic than others.

See the Office Action, page 11, lines 5-11. The Examiner's contention that CpG oligonucleotides may not have identical immunostimulatory activities even though they share the same mechanism of action is correct. However, as discussed above in paragraph 13, the fact that CpG oligonucleotides may have quantitative differences in immunostimulatory activities is irrelevant to the issue at hand, since it is not expected that there are qualitative differences, *i.e.*, all CpG oligonucleotides are expected to have <u>some</u> immunostimulatory activity, and thus are reasonably predicted to exhibit synergy in immune adjuvant activity with QS saponins.

18. With respect to the genus of QS saponins, the Examiner states in the Office Action in support of his position that Kensil I (Exhibit 5) showed that "QS-7, -17, -18, and -21 were structurally different" and Kensil *et al.*, 1991, J. Immunol. 146:431 (attached hereto as

Exhibit 13, "Kensil III") "taught QS-7, -17, -18, and -21 induced different antibody responses (page 434, Fig. 3, Tables 1 and 2).... Therefore, QS-7, -17, -18, and -21 have different structures and induce different immune responses." See the Office Action, page 13, lines 2-6. The Examiner's statement is incorrect and/or misleading with respect to what was taught in Kensil III. Firstly, Figure 3 of Kensil III showed that all four QS saponins, *i.e.*, QS-7, -17, -18, and -21, induced similar antibody (IgG) titers (with overlapping error bars) in mice. Secondly, Table 1 of Kensil III demonstrated that all four QS saponins, *i.e.*, QS-7, -17, -18, and -21, induced IgG2a titers, at levels higher than other adjuvants used. It is not common for adjuvants to induce IgG2a responses. Therefore, the ability of four structurally related QS saponins to induce an IgG2a response is further proof of the similarities in their immune adjuvant activity. Additionally, the Examiner references Table 2 of Kensil III, as showing different antibody responses. However, Table 2 of Kensil III relates to saponin toxicity, not adjuvant activity. Moreover, the fact that QS-7, -17, -18, and -21 have different structures and may induce antibody responses with small quantitative differences is irrelevant to the issue at hand. Firstly, the Examiner's statement ignores the fact that those QS saponins have a common structure that gives rise to their common immune adjuvant activity (see ¶¶ 7-11, hereinabove). Secondly, while small quantitative differences in their immune adjuvant activity may exist, all QS saponins are expected to have qualitatively similar immunostimulatory activity, and thus are reasonably predicted to exhibit synergy in immune adjuvant activity with CpG oligonucleotides. The point is that there are specific structural components, such as a triterpene backbone, a 2,3, glucuronic acid carboxyl group, and an acyl group at certain positions, shared by the QS saponins, that are required for or influence their immune adjuvant activity. It is irrelevant to the issue at hand that each saponin of the genus differs apart from these common structural features.

19. The Examiner further alleges that "[a]pplicants have not shown that the expected immune response obtained using oligonucleotide 1634 or 1758 alone plus the expected immune response obtained using QS-21 alone is less than the immune response obtained when oligonucleotide 1643 or 1758 is combined with QS-21." This statement is incorrect. Example 1 (page 20) and Figure 1 of the '941 application demonstrate that the immune response elicited by oligonucleotide 1758 in combination with QS-21 is more than the additive effects of using oligonucleotide 1758 alone or QS-21 alone with comparable dosages (see *e.g.*, 10 μg QS-21 and

50 μg CpG). Example 2 (page 21) and Example 3 (pages 21-22) of the '941 application demonstrate the same point.

20. I hereby declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: April 23, 2004

Dr. Charlotte Kensil

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1. Saponin Adjuvant.
   Inventors: **Charlotte A. Kensil** and Dante J. Marciani
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number: 5,057,540
   Issued: Oct. 15, 1991

2. Methods for Enhancing Drug Delivery with Modified Saponins
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number: 5,273,965
   Issued: Dec. 28, 1993

3. Vaccine Comprising Recombinant Feline Leukemia Antigen and Saponin Adjuvant
   Inventors: Gerald Beltz, Dante J. Marciani, C.-H. Hung, and **Charlotte A. Kensil**
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number: 5,352,449
   Issued: Oct. 4, 1994

4. Modified Saponins for Enhancing Drug Delivery
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number: 5,443,829
   Issued: Aug. 22, 1995

5. Saponin-Antigen Conjugates and the Use There of
   Inventors: **Charlotte A. Kensil,** Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number 5,583,112
   Issued: Dec. 10, 1996

6. Drug Delivery Enhancement via Modified Saponin
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, Dante J. Marciani, and Joanne Recchia
   Assignee: Antigenics Inc., Framingham, MA
   Patent Number 5,650,398
   Issued: July 22, 1997

7. Saponin Adjuvant Composition
   Inventor: **Charlotte A. Kensil**
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   Patent Number 6,231,859 B1
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(57) Abstract

The present invention relates to adjuvant compositions which are suitable to be used in vaccines. In particular, the adjuvant compositions of the present invention comprises a saponin and an immunostimulatory oligonucleotide, optionally with a carrier. Also provided by the present invention are vaccines comprising the adjuvants of the present invention and an antigen. Further provided are methods of manufacture of the adjuvants and vaccines of the present invention and their use as medicaments. Methods of treating an individual susceptible to or suffering from a disease by the administration of the vaccines of the present invention are also provided.

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Vaccines

The present invention relates to novel adjuvant compositions for use in vaccines. In particular, the adjuvant compositions of the present invention comprise a combination of saponin and an immunostimulatory oligonucleotide, said combination optionally further comprising a carrier. Also provided by the present invention are vaccines comprising the adjuvant compositions of the present invention and at least one antigen. Further provided are methods of manufacture of the adjuvant compositions and vaccines of the present invention and their use as medicaments. Additionally, the present invention provides methods of treating an individual susceptible to or suffering from a disease by the parenteral or mucosal administration of the vaccines of the present invention.

Immunostimulatory oligonucleotides containing unmethylated CpG dinucleotides ("CpG") and are known in the art as being adjuvants when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al.*, *J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the dinucleotide CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequence containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Although other unmethylated CpG containing sequences not having this consensus sequence have now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (PCT Publication No. WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al., Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Saponins are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). Saponins are steroid or triterpene glycosides widely distributed in the plant and marine animal kingdoms. Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating cholesterol. When saponins are near cell membranes they create pore-like structures in the membrane which cause the membrane to burst. Haemolysis of erythrocytes is an example of this phenomenon, which is a property of certain, but not all, saponins.

Saponins are known as adjuvants in vaccines for systemic administration. The adjuvant and haemolytic activity of individual saponins has been extensively studied in the art (Lacaille-Dubois and Wagner, *supra*). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1.

Particulate structures, termed Immune Stimulating Complexes (ISCOMS), comprising fractions of Quil A are haemolytic and have been used in the manufacture of vaccines (Morein, B., EP 0 109 942 B1). These structures have been reported to have adjuvant activity (EP 0 109 942 B1; WO 96/11711).

The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No.5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J. Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Other saponins which have been used in systemic vaccination studies include those derived from other plant species such as Gypsophila and Saponaria (Bomford *et al.*, Vaccine, 10(9):572-577, 1992).

Saponins are also known to have been used in mucosally applied vaccine studies, which have met with variable success in the induction of immune responses. Quil-A saponin has previously been shown to have no effect on the induction of an immune response when antigen is administered intranasally (Gizurarson *et al.* 1994. Vaccine Research 3, 23-29). Whilst, other authors have used this adjuvant with success (Maharaj *et al.*, *Can.J.Microbiol*, 1986, 32(5):414-20; Chavali and Campbell, Immunobiology, 174(3):347-59). ISCOMs comprising Quil A saponin have been used in intragastric and intranasal vaccine formulations and exhibited adjuvant activity (McI Mowat *et al.*, 1991, Immunology, 72, 317-322; McI Mowat and Donachie, Immunology Today, 12, 383-385).

QS21, the non-toxic fraction of Quil A, has also been described as an oral or intranasal adjuvant (Sumino et al., *J.Virol.*, 1998, 72(6):4931-9; WO 98/56415).

The use of other saponins in intranasal vaccination studies has been described. For example, *Chenopodium quinoa* saponins has been used in both intranasal and intragastric vaccines (Estrada *et al., Comp. Immunol. Microbiol. Infect. Dis.*, 1998, 21(3):225-36).

The present invention relates to the surprising finding that immunostimulatory oligonucleotides (CpG) and saponin combinations are extremely potent adjuvants. Accordingly, there is provided an adjuvant composition comprising a combination of saponin and an immunostimulatory oligonucleotide. Preferably, the adjuvants of the present invention may further comprise a carrier. In a preferred form of the present invention the saponin and oligonucleotides in the adjuvant and vaccine compositions act synergistically in the induction of antigen specific antibody and are potent in the induction of immune responses conventionally associated with the Th1-type immune system. Accordingly, the adjuvant combinations are not only suitable for immunoprophylaxis of diseases, but also surprisingly for immunotherapy of diseases such as persistant viral, bacterial or parasitic infections, and also chronic disorders such as cancer.

The preferred oligonucleotides for use in adjuvants or vaccines of the present invention preferably contain two or more dinucleotide CpG motifs separated by at least three, more preferably at least six or more nucleotides. The oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention including oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

Examples of preferred oligonucleotides have the following sequences. The sequences preferably contain phosphorothioate modified internucleotide linkages.

OLIGO 1(SEQ ID NO:1): TCC ATG ACG TTC CTG ACG TT (CpG 1826)

OLIGO 2 (SEQ ID NO:2): TCT CCC AGC GTG CGC CAT (CpG 1758)

OLIGO 3(SEQ ID NO:3): ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

OLIGO 4 (SEQ ID NO:4): TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)

OLIGO 5 (SEQ ID NO:5): TCC ATG ACG TTC CTG ATG CT (CpG 1668)

Alternative CpG oligonucleotides may comprise the preferred sequences above in that they have inconsequential deletions or additions thereto.

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

The oligonucleotides utilised in the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide bond in the oligonucleotide is phosphorodithioate, or more preferably phosphorothioate bond, although phosphodiesters are within the scope of the present invention. Oligonucleotide comprising different internucleotide linkages are contemplated, e.g. mixed phosphorothioate phophodiesters. Other internucleotide bonds which stabilise the oligonucleotide may be used.

The saponins which may be used in the adjuvant combinations of the present invention include those derived from the bark of *Quillaja Saponaria Molina*, termed Quil A, and fractions thereof, described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. Particularly preferred fractions of Quil A are QS21, QS7, and QS17.

β-Escin is another preferred haemolytic saponins for use in the adjuvant compositions of the present invention. Escin is described in the Merck index (12th ed: entry 3737) as a mixture of saponins occurring in the seed of the horse chestnut tree, Lat: *Aesculus hippocastanum*. Its isolation is described by chromatography and purification (Fiedler, *Arzneimittel-Forsch*. 4, 213 (1953)), and by ion-exchange resins (Erbring *et al*., US 3,238,190). Fractions of escin, αand β, have been purified and shown to be

biologically active (Yoshikawa M, et al. (*Chem Pharm Bull* (Tokyo) 1996 Aug;44(8):1454-1464)). β-escin is also known as aescin.

Another preferred haemolytic saponin for use in the present invention is Digitonin. Digitonin is described in the Merck index (12th Edition, entry 3204) as a saponin, being derived from the seeds of *Digitalis purpurea* and purified according to the procedure described Gisvold *et al.*, *J.Am.Pharm.Assoc.*, 1934, 23, 664; and Ruhenstroth-Bauer, *Physiol.Chem.*, 1955, 301, 621. Its use is described as being a clinical reagent for cholesterol determination.

The adjuvant combinations of the present invention may further comprise a carrier, such that the saponin or CpG, or both, may be associated with a particulate carrier entity to enhance the adjuvanticity of the combination. Particularly preferred systemic vaccines, for example, comprise a carrier molecule.

The CpG used in the adjuvant combinations of the present invention may be in free solution or may be complexed to particulate carriers such as mineral salts (for example, but not restricted to, aluminium or calcium salts), liposomes, ISCOMs, emulsions (oil in water, water in oil, water in oil in water), polymers (such as, but not restricted to polylactic, polyglycolic, polyphosphazine, polyaminoacid, alginate, chitosan) or microparticles. Preferably said carriers are cationic. The vaccines of the present invention further comprise an antigen which may be associated with the CpG-carrier complex, or may not be associated with the CpG-carrier complex. In this case, the antigen may be free suspension or associated with a separate carrier.

The saponins forming part of the present invention may be separate in the form of micelles, or may be in the form of large ordered structures such as ISCOMs (EP 0 109 942 B1) or liposomes (WO 96/33739) when formulated with cholesterol and lipid, or in the form of an oil in water emulsion (WO 95/17210). The saponins may preferably be associated with a metallic salt, such as aluminium hydroxide or aluminium phosphate (WO 98/15287). Alternatively the saponin may be associated with a particulate carrier such as chitosan. The saponin may also be in a dry state such as a

powder. The final formulations in the form as they are administered to the mucosal surface of the vaccinee are preferably haemolytic in nature. The saponin may or may not be associated physically with the antigen either through direct linkage or by co-interaction with the same particulate carrier molecule (GB9822712.7; WO 98/16247). The CpG and saponin in the adjuvants or vaccines of the present invention may themselves be separate or associated. For example, the CpG and saponin may be in free suspension or may be associated via a carrier, more preferably a particulate carrier such as aluminium hydroxide or by a cationic liposome or ISCOM.

A preferred adjuvant combination according to the present invention is composed of one or more CpG oligonucleotides containing at least 3, preferably at least 6 nucleotides between two adjacent CG motifs, together with QS21 and a particulate carrier selected from the group comprising an oil-in-water emulsion or DQ. Most preferably, the adjuvant combination comprises CpG 2006 (SEQ ID NO: 4), or CpG 1758 (SEQ ID NO: 2) or CpG 1826 (SEQ ID NO: 1) mixed with QS21, and a particulate carrier selected from the group comprising an oil-in-water emulsion or DQ. Accordingly, particularly preferred vaccines, for example, comprise such adjuvant combinations and an antigen. The preferred vaccine of the present invention is used to generate systemic immune responses after administration to an individual through the systemic route.

The adjuvant combinations of the present invention may be used as both systemic or mucosal adjuvant. In a particular form of the invention there is provided a systemic vaccine to be administered through the systemic or parenteral route such as intramuscular, intradermal, transdermal, subcutaneous, intraperitoneal or intravenous administration. A preferred route of administration is via the transdermal route, for example by skin patches.

The systemic vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to, or suffering from disease, by means of administering said vaccine by intramuscular, intraperitoneal, intradermal, transdermal, intravenous, or subcutaneous administration. Methods of systemic administration of the vaccine

preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or needleless pressure liquid jet device (US 4,596,556; US 5,993,412), or transdermal patches (WO 97/48440; WO 98/28037). The present invention may also be used to enhance the immunogenicity of antigens applied to the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037). The present invention therefore provides a delivery device for systemic administration, pre-filled with the vaccine or adjuvant compositions of the present invention. Accordingly there is provided a method for inducing an immune response in an individual, comprising the administration of a vaccine comprising an antigen and immunostimulatory oligonucleotide, a saponin, and a carrier, to the individual, wherein the vaccine is administered via the parenteral or systemic route. Preferred methods of inducing an immune response comprises the administration of a vaccine comprising an oligonucleotide of SEQ ID NO: 1, 2, 3, 4 or 5, with a saponin derived from QuilA, such as QS21, and a carrier, such as an oil in water emulsion, a cholesterol containing liposome or alum.

Alternatively the vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to, or suffering from disease, by means of administering said vaccine via a mucosal route, such as the oral/alimentary or nasal route. Alternative mucosal routes are intravaginal and intra-rectal. The preferred mucosal route of administration is *via* the nasal route, termed intranasal vaccination. Methods of intranasal vaccination are well known in the art, including the administration of a droplet, spray, or dry powdered form of the vaccine into the nasopharynx of the individual to be immunised. Nebulised or aerosolised vaccine formulations also form part of this invention. Enteric formulations such as gastro resistant capsules and granules for oral administration, suppositories for rectal or vaginal administration also form part of this invention.

The adjuvant combinations of the present invention, represent a class of mucosal adjuvants suitable for application in humans to replace systemic vaccination by mucosal vaccination. In a preferred form of the present invention pure saponins such as Quil A, or derivatives thereof, including QS21; Escin; Digitonin; or *Gypsophila* or

Chenopodium quinoa saponins in combination with immunostimulatory oligonucleotides may be used as adjuvants for the mucosal administration of antigens to achieve a systemic immune response.

The adjuvant combinations of the present invention are used in the formulation of vaccines, which vaccines may be administered via the systemic or mucosal route. Preferably, when the vaccines are used for mucosal administration the adjuvant combination comprises a haemolytic saponin.

For mucosal administration preferably the composition of the invention comprise a haemolytic saponin. Haemolytic saponin, or saponin preparation, within the meaning of this invention is to be determined with reference to the following assay.

1. Fresh blood from guinea pigs is washed with phosphate buffered saline (PBS) 3 times in a desk-top centrifuge. After resuspension to the original volume the blood is further diluted 10 fold in PBS.

2. 50 µl of this blood suspension is added to 800 µl of PBS containing two-fold dilutions of surfactant or saponin.

3. After 8 hours the haemolysis is assessed visually or by measuring the optical density of the supernatant. The presence of a red supernatant, which absorbs light at 570 nm indicates the presence of haemolysis.

4. The results are expressed as the concentration of the first saponin dilution at which hemolysis no longer occurs.

For the purposes of this invention the saponin adjuvant preparation is haemolytic if it lyses the erythrocytes at a concentration of less than 0.1%. As means of reference, substantially pure samples of QuilA, QS21, QS7, Digitonin, and β-escin are all haemolytic saponins as defined in this assay. Within the inherent experimental variability of such a biological assay, the saponins of the present invention preferably have a haemolytic activity, of approximately between 0.5-0.00001%, more preferably between 0.05-0.00001%, even more preferably between 0.005-0.00001%, and most preferably between 0.001-0.0004%. Ideally, said saponins should have a haemolytic activity similar (*i.e.* within a ten-fold difference) to that of QS21.

The vaccines of the present invention may also be administered via the oral route. In such cases the pharmaceutically acceptable excipient may also include alkaline buffers, or enteric capsules or microgranules. The vaccines of the present invention may also be administered by the vaginal route. In such cases, the pharmaceutically acceptable excipients may also include emulsifiers, polymers such as CARBOPOL®, and other known stabilisers of vaginal creams and suppositories. The vaccines of the present invention may also be administered by the rectal route. In such cases the excipients may also include waxes and polymers known in the art for forming rectal suppositories.

Preparations of more than one saponin in the adjuvant combinations of the present invention are also form part of the present invention. For example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin. Additionally, the compositions of the present invention may comprise combinations of more than one immunostimulatory oligonucleotide.

In a similar embodiment of the present invention the CpG/saponin combinations for both systemic and mucosal administration may be further combined with other adjuvants including. Monophosphoryl Lipid A and its non-toxic derivative 3-de-O-acylated monophosphoryl lipid A. Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamelar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol^{R} to increase viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

3 De-O-acylated monophosphoryl lipid A is a well known adjuvant manufactured by Ribi Immunochem, Montana. It can be prepared by the methods taught in GB 2122204B. A preferred form of 3 De-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2μm in diameter (EP 0 689 454 B1). Particularly preferred adjuvants are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, WO 98/56414), or 3D-MPL formulated with other carriers (EP 0 689 454 B1).

Preferably the vaccine formulations of the present invention contain an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, ..), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (whole live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof), or derived from bacterial pathogens such as *Neisseria spp*, including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *S. pyogenes* (for example M proteins or fragments thereof; C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; H. ducreyi; Moraxella spp, including M*

catarrhalis, also known as Branhamella catarrhalis (for example high and low molecular weight adhesins and invasins*); Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or –C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof*); Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof*), C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.,* including *B. anthracis* (for example botulinum toxin and derivatives thereof*); Corynebacterium spp.,* including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.,* including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB*), B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.,* including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis*; Rickettsia spp,* including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins*), C. psittaci; Leptospira spp.,* including *L. interrogans; Treponema spp.,* including *T. pallidum* (for example the rare outer membrane proteins*), T. denticola, T. hyodysenteriae;* or

derived from parasites such as *Plasmodium spp.*, including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni*, or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans.*

Other preferred specific antigens for *M. tuberculosis* are for example Tb Ra12, Tb H9, Tb Ra35, Tb38-1, Erd 14, DPV, MTI, MSL, mTTC2 and hTCC1 (WO 99/51748). Proteins for *M. tuberculosis* also include fusion proteins and variants thereof where at least two, preferably three polypeptides of *M. tuberculosis* are fused into a larger protein. Preferred fusions include Ra12-TbH9-Ra35, Erd14-DPV-MTI, DPV-MTI-MSL, Erd14-DPV-MTI-MSL-mTCC2, Erd14-DPV-MTI-MSL, DPV-MTI-MSL-mTCC2, TbH9-DPV-MTI (WO 99/51748).

Most preferred antigens for Chlamydia include for example the High Molecular Weight Protein (HWMP) (WO 99/17741), ORF3 (EP 366 412), and putative membrane proteins (Pmps). Other Chlamydia antigens of the vaccine formulation can be selected from the group described in WO 99/28475.

Preferred bacterial vaccines comprise antigens derived from *Streptococcus spp*, including *S. pneumoniae* (for example capsular polysaccharides and conjugates thereof, PsaA, PspA, streptolysin, choline-binding proteins) and the protein antigen Pneumolysin (Biochem Biophys Acta, 1989, 67, 1007; Rubins et al., Microbial Pathogenesis, 25, 337-342), and mutant detoxified derivatives thereof (WO 90/06951; WO 99/03884). Other preferred bacterial vaccines comprise antigens derived from *Haemophilus spp., including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae,* for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464) or multiple copy varients or fusion proteins thereof.

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS1, PreS2 S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474. In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD2t as hereinabove defined.

In a preferred embodiment of the present invention vaccines containing the claimed adjuvant comprise antigen derived from the Human Papilloma Virus (HPV) considered to be responsible for genital warts (HPV 6 or HPV 11 and others), and the HPV viruses responsible for cervical cancer (HPV16, HPV18 and others).

Particularly preferred forms of genital wart prophylactic, or therapeutic, vaccine comprise L1 particles or capsomers, and fusion proteins comprising one or more antigens selected from the HPV 6 and HPV 11 proteins E6, E7, L1, and L2.

The most preferred forms of fusion protein are: L2E7 as disclosed in WO 96/26277, and proteinD(1/3)-E7 disclosed in GB 9717953.5 (PCT/EP98/05285).

A preferred HPV cervical infection or cancer, prophylaxis or therapeutic vaccine, composition may comprise HPV 16 or 18 antigens. For example, L1 or L2 antigen monomers, or L1 or L2 antigens presented together as a virus like particle (VLP) or the L1 alone protein presented alone in a VLP or caposmer structure. Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, and WO93/02184.

Additional early proteins may be included alone or as fusion proteins such as E7, E2 or preferably E5 for example; particularly preferred embodiments of this includes a VLP comprising L1E7 fusion proteins (WO 96/11272).

Particularly preferred HPV 16 antigens comprise the early proteins E6 or E7 in fusion with a protein D carrier to form Protein D - E6 or E7 fusions from HPV 16, or combinations thereof; or combinations of E6 or E7 with L2 (WO 96/26277).

Alternatively the HPV 16 or 18 early proteins E6 and E7, may be presented in a single molecule, preferably a Protein D- E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D - E6 or Protein D - E7 fusion protein or Protein D E6/E7 fusion protein.

The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 31 or 33.

Vaccines of the present invention further comprise antigens derived from parasites that cause Malaria. For example, preferred antigens from *Plasmodia falciparum* include RTS,S and TRAP. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P.falciparum* linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in the International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S. TRAP antigens are described in the International Patent Application No. PCT/GB89/00895, published under WO 90/01496. A preferred embodiment of the present invention is a Malaria vaccine wherein the antigenic preparation comprises a combination of the RTS,S and TRAP antigens. Other plasmodia antigens that are likely candidates to be components of a multistage Malaria vaccine are *P. faciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in Plasmodium spp.

The formulations may also contain an anti-tumour antigen and be useful for the immunotherapeutic treatment of cancers. For example, the adjuvant formulation finds utility with tumour rejection antigens such as those for prostrate, breast, colorectal, lung, pancreatic, renal or melanoma cancers. Exemplary antigens include MAGE 1 and MAGE 3 or other MAGE antigens (for the treatment of melanoma), PRAME, BAGE, or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al. (1997), Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other tumour-specific antigens are suitable for use with the adjuvants of the present invention and include, but are not restricted to tumour-specific gangliosides, Prostate specific antigen (PSA) or Her-2/neu, KSA (GA733), PAP, mammaglobin, MUC-1, carcinoembryonic antigen (CEA). Accordingly in one aspect of the present invention there is provided a vaccine comprising an adjuvant composition according to the invention and a tumour rejection antigen.

It is a particularly preferred aspect of the present invention that the vaccines comprise a tumour antigen; such vaccines are surprisingly potent in the therapy of cancer such as prostrate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers. Accordingly, the formulations may contain tumour-associated antigen, as well as antigens associated with tumour-support mechanisms (e.g. angiogenesis, tumour invasion). Additionally, antigens particularly relevant for vaccines in the therapy of cancer also comprise Prostate-specific membrane antigen (PSMA), Prostate Stem Cell Antigen (PSCA), tyrosinase, survivin, NY-ESO1, prostase, PS108 (WO 98/50567), RAGE, LAGE, HAGE. Additionally said antigen may be a self peptide hormone such as whole length Gonadotrophin hormone releasing hormone (GnRH, WO 95/20600), a short 10 amino acid long peptide, useful in the treatment of many cancers, or in immunocastration.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from *Borrelia sp.*. For example, antigens may

include nucleic acid, pathogen derived antigen or antigenic preparations, recombinantly produced protein or peptides, and chimeric fusion proteins. In particular the antigen is OspA. The OspA may be a full mature protein in a lipidated form virtue of the host cell (E.Coli) termed (Lipo-OspA) or a non-lipidated derivative. Such non-lipidated derivatives include the non-lipidated NS1-OspA fusion protein which has the first 81 N-terminal amino acids of the non-structural protein (NS1) of the influenza virus, and the complete OspA protein, and another, MDP-OspA is a non-lipidated form of OspA carrying 3 additional N-terminal amino acids.

Vaccines of the present invention may be used for the prophylaxis or therapy of allergy. Such vaccines would comprise allergen specific (for example Der p1) and allergen non-specific antigens (for example peptides derived from human IgE, including but not restricted to the stanworth decapeptide (EP 0 477 231 B1)).

Vaccines of the present invention may also be used for the prophylaxis or therapy of chronic disorders others than allergy, cancer or infectious diseases. Such chronic disorders are diseases such as atherosclerosis, and Alzheimer.

Antigens relevant for the prophylaxis and the therapy of patients susceptible to or suffering from Alzheimer neurodegenerative disease are, in particular, the N terminal 39 –43 amino acid fragment (Aβ) of the amyloid precursor protein and smaller fragments. This antigen is disclosed in the International Patent Application No. WO 99/27944 – (Athena Neurosciences).

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-500 μg, preferably 1-100μg, most preferably 1 to 50μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in vaccinated subjects. Following an initial vaccination, subjects may receive one or

several booster immunisation adequately spaced. Such a vaccine formulation may be applied to a mucosal surface of a mammal in either a priming or boosting vaccination regime; or alternatively be administered systemically, for example *via* the transdermal, subcutaneous or intramuscular routes.

The amount of CpG or immunostimulatory oligonucleotides in the adjuvants or vaccines of the present invention is generally small, but depending on the vaccine formulation may be in the region of 1-1000μg per dose, preferably 1-500μg per dose, and more preferably between 1 to 100μg per dose.

The amount of saponin for use in the adjuvants of the present invention may be in the region of 1-1000μg per dose, preferably 1-500μg per dose, more preferably 1-250μg per dose, and most preferably between 1 to 100μg per dose. The ratio of CpG:saponin (w/w) will, therefore, be in the range of 1:1000 to 1000:1, and will typically be in the range of 1:100 to 100:1, and preferably in the range of 1:10 to 1:1 or 1:1 to 10:1, and most preferably 1:1, 4:1 or 10:1.

The formulations of the present invention maybe used for both prophylactic and therapeutic purposes. Accordingly, there is provided the use of a combination of a saponin and a CpG molecule in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer and other non-chronic disorders. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease or cancer, or allergy, or autoimmune disease. In a further aspect of the present invention there is provided a vaccine or adjuvant combination, comprising a saponin and CpG, as herein described for use as a medicament. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from a wide variety of sources. For example, antigens may include human, bacterial, or viral nucleic acid, pathogen derived antigen

or antigenic preparations, tumour derived antigen or antigenic preparations, host-derived antigens, including peptides derived from IgE, such as the histamine releasing decapeptide of IgE (known as the Stanworth decapeptide), recombinantly produced protein or peptides, and chimeric fusion proteins.

There is provided by the present invention a systemic vaccine composition comprising an antigen, a saponin and an immunostimulatory oligonucleotide. Accordingly, there is provided a method of treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially described herein through the systemic route of said individual. Also provided is a method to prevent an individual from contracting a disease selected from the group comprising infectious bacterial and viral diseases, parasitic diseases, prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; non-cancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a composition as substantially described herein through the systemic route of said individual.

Alternatively, there is provided by the present invention a mucosal vaccine composition comprising an antigen, and a haemolytic saponin. Accordingly, there is provided a method of treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially herein described to a mucosal surface of said individual.

Furthermore, there is described a method of inducing a systemic antigen specific immune response in a mammal, comprising administering to a mucosal surface of said mammal a composition comprising an antigen and a haemolytic saponin. Further there is provided a method of manufacture of a vaccine or adjuvant are also provided, comprising taking a saponin and taking a CpG molecule and admixing them with an antigen.

Examples of suitable pharmaceutically acceptable excipients for use in the combinations of the present invention include water, phosphate buffered saline, isotonic buffer solutions.

FIGURE LEGENDS

Figure 1: OspA specific IgG titres 14 days after the nasal boosting.

Figure 2: OspA specific LA2 titres 14 days after the nasal boosting.

Figure 3: serum Flu strain specific IgG titres 14 days after the nasal boosting.

Figure 4: serum Flu strain specific serum HemAgglutination Inhibition (HAI) titres 14 days after the nasal boosting.

Figure 5: OspA specific LA2 titres in mice

Figure 6: gp120-specific lymphoproliferation activity of spleen cells from immunized mice. The antigen-specific activity is expressed as SI for different antigen concentrations for all 4 experimental groups.

Figure 7: HBsAg-specific CTL activity of spleen cells from immunized mice. Effector cell activity was assessed by examining ^{51}Cr release of P815 cells (open circles) or *s*-transfected P815 cells (closed circles).

Figure 8: HBsAg-specific antibody responses in immunized mice. Specific antibody titers (expressed as EU/ml) and isotype profiles were evaluated using ELISA tests. Values from pooled sera are shown in the table, and isotype distributions are also depicted in a graphic.

Figure 9: HBSAg- and gp120-specific lymphoproliferation activity of spleen cells from immunized mice. The antigen-specific activity is expressed as SI for different antigen concentrations for all 4 experimental groups.

Figure 10: HBsAg- and gp120-specific CTL activity of spleen cells from immunized mice. Effector cell activity was assessed by examining ^{51}Cr release of control P815

cells (open symbols) or P815 cells displaying an HBsAg or gp120 CTL epitope (closed symbols).

Figure 11: Gp120-specific and HbsAg-specific antibody responses in immunized mice. Specific antibody titers (expressed in µg/ml) (Figure 11A) and isotype profiles were evaluated using ELISA tests. Values from pooled sera are shown in the table, and isotype distributions are also depicted in a graphic. Figure 11B shows the isotype pattern of gp120-specific antibodies.

Figure 12: Evolution of the mean tumour growth per groups of 10 animals over time.

The present invention is illustrated by, but not restricted to, the following examples.

EXAMPLE 1 ***The use of QS21 and CpG for the intranasal boosting of systemic antibodies to Lipo-OspA***

In this example we investigated whether lytic saponins such as QS21 and immunostimulants such as CpG were able to enhance in a synergistic fashion systemic immunological responses to an intranasal boosting vaccination of mice. Female Balb/c mice (5 animals per group), aged 8 weeks, were immunized intramuscularly with lipo-OspA (1µg) formulated onto alum (50 µg). After 3 months, the mice were boosted intranasally (under anesthesia) with 10 µl of solution (5 µl per nostril, delivered as droplets by pipette) containing 5 µg lipo-OspA in either A: PBS; B: 20 µg CpG 1001 (TCC ATG AGC TTC CTG ACG TT, Krieg 1826); C: 5 µg QS21 (obtained from Cambridge Biotech, USA); D: 20 µg CpG 1001 + 5 µg QS21; or, E: by intra muscular injection of 1 µg lipo-OspA adsorbed onto alum (50 µg).
Figures 1 and 2 show the OspA specific IgG titres and LA2 titres 14 days after the nasal boosting.

Methods

ELISA for the measurement of OspA-specific serum IgG in mice:

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 µl/well of 1 µg/ml OspA diluted in PBS (in rows B to H of plate), or with 50 µl of 5 µg/ml purified goat anti-mouse Ig (Boerhinger), in PBS (row A). Free sites on the plates are blocked (1 hour, 37°C) using saturation buffer : PBS containing 1%BSA, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20), and 4% Normal Bovine Serum (NBS). Then, serial 2-fold dilutions of IgG isotype mixture, diluted in saturation buffer (50 µl per well) and added as a standard curve (mixture of mouse monoclonal antibodies IgG1, IgG2a and IgG2b from Sigma, starting at 200 ng/ml and put in row A), and serum samples (starting at a 1/100 dilution and put in rows B to H) are incubated for 1hr 30mins at 37°C. The plates are then washed (×3) with washing buffer (PBS, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20)). Then, biotinylated goat anti-

mouse IgG (Amersham) diluted 1/5000 in saturation buffer are incubated (50 µl/well) for 1hr 30mins, at 37°C. After 3 washings, and subsequent addition of streptavidin-horseradish peroxidase conjugate (Amersham), plates are washed 5 times and incubated for 20 min at room temperature with 50 µl/well of revelation buffer (OPDA 0.4 mg/ml (Sigma) and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). Revelation is stopped by adding 50 µl/well H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. Antibody titers are calculated by the 4 parameter mathematical method using SoftMaxPro software.

Inhibition assay for the measurement of serum LA2-like Antibody titres to lipo-OspA

Antibody titres in the vaccinees were studied with respect to their LA2-like specificity. LA2 is a murine monoclonal antibody which recognizes a conformational OspA epitope at the surface of the bacteria and has been shown to be able to kill B. burgdorferi in vitro, as well as to protect mice against a challenge with laboratory-grown spirochete (Schaible UE et al. 1990. Proc Natl Acad Sci USA 87:3768-3772). Moreover, LA-2 mab has been shown to correlate with bactericidal antibodies, and studies on human sera showed also a good correlation between the total anti-OspA IgG titers and the LA-2 titers (as measured by ELISA).

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 µl/well of 0.5µg/ml lipo OspA diluted in PBS. Free sites were blocked with saturation buffer for 1hr at 37°C with (100 µl/well of saturation buffer: PBS/ BSA 1%/ Tween 20 0.1%/ NBS 4%). Serial 2-fold dilutions of LA2 monoclonal Ab (mAb) starting at 4 µg/ml were diluted in saturation buffer (50 µl per well) to form a standard curve. Dilutions of serum samples from the vaccinees (starting at a 1/10 dilution) were also added and the plates incubated for 2hrs at 37°C. The plates were washed after incubation 3 times with PBS/ TWEEN 20 (0.1%). LA2 mAb-peroxidase conjugate (1/10,000) diluted in saturation buffer was added to each well (50 µl/well) and incubated for 1hr at 37°C. After 5 washings, plates are incubated for 20 min at room temperature (in darkness) with 50 µl/well of revelation buffer (OPDA 0.4 mg/ml and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). The reaction and colour formation was stopped with H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. LA2-like Ab titers are calculated by the 4 parameter mathematical method using

SoftMaxPro software. LA2-like antibody titres were determined by comparison with the standard curve.

Results

CpG as well as QS21 improve significantly the intranasal boosting of systemic antibodies to Lipo-OspA. Moreover, when both adjuvants are combined, a synergistic effect on those responses is clearly demonstrated, especially in term of LA2 antibodies. Humoral responses elicited in the presence of QS21 and CpG are significantly higher than those induced by the parenteral booster. Taken together, these results show clearly the potential of intranasal formulations combining a lytic saponin and an immunostimulant.

EXAMPLE 2. ***Synergistic combination of QS21 and CpG for enhancing the intranasal boosting of systemic antibodies to influenza virus***

In this example we investigated whether haemolytic saponins such as QS21 (see example) and immunostimulants such as CpG were able to enhance in a synergistic fashion the intranasal boost of systemic antibodies in mice primed intranasally with inactivated whole influenza virus.

Female Balb/c mice (10 animals per group), aged 8 weeks, were primed intranasally with β-propiolactone inactivated trivalent whole influenza virus (A/Beijing/262/95; A/Johannesburg/33/94; B/Panama/45/90; 5 μg HA / strain) for mimicking the natural priming occurring in humans. After 28 days, the mice were boosted intranasally (under anesthesia) with 20 μl of solution (10 μl per nostril, delivered as droplets by pipette) containing 1.5 μg HA / strain of β-propiolactone inactivated trivalent whole influenza virus (same strains as in the priming immunization) in either A: PBS; B: 50 μg CpG (TCG TCG TTT TGT CGT TTT GTC GTT, Krieg 2006); C: 4.5 μg QS21 (obtained from Cambridge Biotech, USA); D: 50 μg CpG + 4.5 μg QS21; or, E: by intra muscular injection of 1.5 μg HA / strain of trivalent split influenza virus (same strains as in the priming immunization). Flu antigens were supplied by SSD GmBH manufacturer (Dresden, Germany).

Figures 3 and 4 show the serum Flu strain specific IgG titres and HemAgglutination

Inhibition (HAI) titres 14 days after the nasal boosting.

Methods

ELISA for the measurement of Anti-influenza IgG titres in mice:

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 µl/well of 1 µg/ml whole influenza virus antigen diluted in PBS (in rows B to H of plate), or with 50 µl of 5 µg/ml purified goat anti-mouse Ig (Boerhinger), in PBS (row A). Free sites on the plates are blocked (1 hour, 37°C) using saturation buffer : PBS containing 1%BSA, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20), and 4% Normal Bovine Serum (NBS). Then, serial 2-fold dilutions of IgG isotype mixture, diluted in saturation buffer (50 µl per well) and added as a standard curve (mixture of mouse monoclonal antibodies IgG1, IgG2a and IgG2b from Sigma, starting at 200 ng/ml and put in row A), and serum samples (starting at a 1/100 dilution and put in rows B to H) are incubated for 1hr 30mins at 37°C. The plates are then washed (×3) with washing buffer (PBS, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20)). Then, biotinylated goat anti-mouse IgG (Amersham) diluted 1/5000 in saturation buffer are incubated (50 µl/well) for 1hr 30mins, at 37°C. After 3 washings, and subsequent addition of streptavidin-horseradish peroxidase conjugate (Amersham), plates are washed 5 times and incubated for 20 min at room temperature with 50 µl/well of revelation buffer (OPDA 0.4 mg/ml (Sigma) and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). Revelation is stopped by adding 50 µl/well H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. Antibody titers are calculated by the 4 parameter mathematical method using SoftMaxPro software.

The Whole influenza virus used for the coating (strain A/Beijing/262/95), inactivated with β-propiolactone (BPL), is supplied by SSD GmBH manufacturer (Dresden, Germany).

HemAgglutination Inhibition (HAI) activity of Flu-specific serum Abs in mice

Sera (25 µl) are first treated for 20 minutes at room temperature (RT) with 100 µl borate 0.5M buffer (pH 9) and 125 µl Dade Behring-purchased kaolin. After centrifugation (30 minutes, 3000 RPM or 860 g), 100 µl supernatant (corresponding

to a 1/10 dilution of the serum) are taken and incubated for 1 hour at 4°C with 0.5% chicken red blood cells. Supernatant is collected after centrifugation for 10 minutes at 3200 RPM (970 g). Both operations are done for eliminating the natural hemagglutinating factors contained in the sera. Then, 25 µl treated-sera are diluted in 25 µl PBS (serial 2-fold dilutions starting at 1/20) in 96 well Greiner plates. BPL inactivated whole virus is added (25 µl / well) at a concentration of 4 Hemagglutination Units (i.e. at a dilution which is 4-fold lower than the last one provoking an agglutination of red blood cells) for 30 minutes at RT under agitation. Chicken red blood cells are then added (25 µl / well) for 1 hour at RT. Plates are finally kept overnight at 4°C before to be read. The HAI titer corresponds to the last serum dilution inhibiting the virus-induced hemagglutination.

Results

CpG as well as QS21 do not improve the intranasal boosting of IgG or HAI antibodies to Flu strains. However, when both adjuvants are combined, a synergistic effect on those responses is clearly demonstrated. The HAI responses elicited in the presence of QS21 and CpG are even similar than those induced by the parenteral booster. These results confirm the potential of intranasal formulations combining a haemolytic saponin and an immunostimulant. They also show that several CpG sequences can be efficient in this context (Krieg 2006 in the present example and Krieg 1826 in the examples 3 and 5).

EXAMPLE 3. *Synergistic combination of β-Escin and CpG for enhancing the intranasal boosting of systemic antibodies to Lipo-OspA*

We assess in the present example the possibility that a synergy similar to that observed between QS21 and CpG could be obtained with other haemolytic saponins (see example) such as β-Escin. The non haemolytic saponin, glycyrrhizic acid, is also tested.

Female Balb/c mice (6 animals per group), aged 8 weeks, were primed intramuscularly with lipo-OspA (1µg) formulated onto alum (50 µg). After 3 months, the mice were boosted intranasally (under anesthesia) with 10 µl of solution (5 µl per

nostril, delivered as droplets by pipette) containing 5 µg lipo-OspA in either A: PBS; B: 50 µg CpG 1001 (TCC ATG AGC TTC CTG ACG TT, Krieg 1826); C: 5 µg β-Escin (purchased from Sigma); D: 50 µg CpG 1001 + 5 µg β-Escin; E: 5 µg glycyrrhizic acid (purchased from Sigma); F: 50 µg CpG 1001 + 5 µg glycyrrhizic acid or, G: by intra muscular injection of 1 µg lipo-OspA adsorbed onto alum (50 µg). Figure 5 shows the OspA specific-LA2 titres 14 days after the nasal boosting.

Methods

The methods are the same as those detailed in Example 1.

Results

β-Escin and CpG act synergistically for enhancing the intranasal boosting of systemic LA2 Abs. This combination elicits more elevated Ab responses than the parenteral booster. On the other hand, such a synergy is not obtained by combining CpG with glycyrrhizic acid.

These results and the previous ones of this patent taken together show the ability of CpG and different haemolytic saponins to adjuvant immune responses in a synergistic fashion.

EXAMPLE 4. *Immunogenicity studies using P. falciparum RTS,S and HIV-1 gp120 formulated with CpG and/or DQS21*

1. Experiment outline

Two mouse immunogenicity studies were conducted to evaluate potential additive or synergistic effects of CpG oligonucleotides (CpG) and QS21. Groups of mice were immunized with RTS,S and gp120 formulated with CpG and QS21 alone or in combination. These adjuvant combinations were also tested in the presence of the carrier Al(OH), or an oil-in-water (o/w) emulsion.

The immunogenicity of the formulations was examined after two parenteral immunizations. Sera were analyzed for the presence of antigen-specific antibodies,

and for the distribution of antibody isotypes. Spleen cells were used to evaluate cell-mediated immune responses. Those cells were tested for the presence of cytotoxic T lymphocytes (CTL) and lymphoproliferative (lymphoproliferation) cells.

Table 1: Groups of mice in **experiment 1**

Group	antigen	adjuvant
1	RTS,S/gp120	CpG/DQS21
2	RTS,S/gp120	DQS21
3	RTS,S/gp120	CpG/DQS21/Al(OH)$_3$
4	RTS,S/gp120	CpG/Al(OH)$_3$

Table 2: Groups of mice in **experiment 2**

Group	antigen	adjuvant
1	RTS,S/gp120	CpG
2	RTS,S/gp120	CpG/DQS21
3	RTS,S/gp120	CpG/QS21/o/w emulsion

2. Formulation

2.1. Experiment 1

Formulation process:

Formulations were prepared three days before each injection. When needed, RTS,S (10 µg) and gp 120 (10µg) were adsorbed on 100 µg of AL(OH)$_3$. When needed, MPL (5 µg) was added and incubated 30 min before buffer addition as a mix of 10-fold concentrated PBS pH 7.4 and H_2O excepted for the group without DQ for which the buffer was PO_4, NaCl 10/150 pH 6.8. After 30 min, if needed, QS21 (5 µg) mixed with liposomes in a weight ratio QS21/cholesterol of 1/5 (referred to as DQ) was added to the formulation. Thirty minutes later, for the formulations with the oligo,

100 µg of CpG was added 30 min prior addition of 50 µg/ml of thiomersal as preservative.

$Al(OH)_3$ + RTSS + gp120 - 1h- MPL -30min- premix -30min- DQ -30min- CpG -30min- Thio

All incubations were carried out at room temperature with agitation.

2.2. Experiment 2

Formulation process:

Formulations are performed simultaneously for both injections. The volume of injection for one mouse is 100 µl. Fifty µg/ml of thiomersal is added as preservative.

Group 1 : RTS,S (10 µg) and gp120 (10 µg) are diluted with H_2O and PBS pH 6.8 for isotonicity. After 5 min., the formulation is adsorbed on CpG 1856 (100 µg).

Group 2 : RTS,S (10 µg) and gp120 (10 µg) are diluted with H_2O and PBS pH 7.4 for isotonicity. After 30 minutes RTS,S and gp120 are adsorbed on DQ (5 µg) . After 30 min. of adsorption, the formulation is adsorbed on CpG 1856 (100 µg).

Group 3 : RTS,S (10 µg) and gp120 (10 µg) are diluted with H_2O and PBS pH 6.8 for isotonicity. After 5 min., the formulation is adsorbed on an o/w emulsion. After 5 min. of adsorption, the formulation is adsorbed on QS21 (5 µg) prior the addition of CpG (100 µg).

3. Immunological methods

Nine (Balb/C x C57Bl/6) F1 mice per group received into the hind footpads 2 x 50 µl vaccine twice at a two-week-interval. Two weeks later sera were obtained to assess antibody responses, and spleen cells were harvested to determine cell-mediated immune responses.

For lymphoproliferation analysis, cells were seeded in quadruplicates in 96-well round-bottomed microtiter plates at a concentration of $2x10^6$ per ml. Cells were

cultured for 72 or 96 hrs in RPMI-1640 supplemented with antibiotics, glutamine and 1 % (v/v) normal mouse serum in the presence of different concentrations of RTS,S or gp120 antigen. Control cells were cultured without antigen. Then the cells were pulsed overnight with 1µCi/well [^{3}H]-thymidine, harvested and the incorporated radioactivity was determined in a beta-counter. Results are expressed as mean counts per minute (cpm).

For CTL analysis cells were cultured for 7 days in 6-well plates in the presence of 10 µg per ml of synthetic peptide pCMI003 (IPQSLDSWWTSL) corresponding to an HBsAg CTL epitope (Schirmbeck et al., 1995) or peptide pCMI007 (GIHIGPGRAFYAARK) representing an gp120 CTL epitope (Casement et al., 1995). At the end of the culture period effector cells were assessed in duplicate for HBsAg-specific cytolytic activity in standard [^{51}Cr]-release assays using control and S-transfected P815 cells. Gp120-specific cytotoxicity was determined by using P815 target cells that were either left untreated or pulsed for 1 hr with peptide pCMI007. Minimum and maximum release were determined with target cells without effector cells and by the addition of 3 % (v/v) Triton X-100, respectively. Results are expressed as % [51Cr]-release (cpm of experimental culture - cpm of spontaneous release / cpm of maximum release - cpm of spontaneous release).

Titration and isotyping of pooled sera was performed in a standard enzyme-linked immunosorbent assay (ELISA) format using plates coated with HbsAg. Sera were diluted in PBS/BSA starting at 1:400. Biotinylated secondary antibodies specific for Ig or the isotypes IgG1, IgG2a and IgG2b followed by a horseradish peroxydase-streptavidin conjugate were used for detection of bound antibodies. ELISA titers were calculated from a reference by SoftmaxPro and expressed in ELISA units (EU/ml). Gp120-specific antibody titers were determined in a standard ELISA using plates coated with gp120 protein. Sera were diluted in PBS/Tween20/BSA starting at 1:100. Biotinylated secondary antibodies specific for Ig or the isotypes IgG1, IgG2a and IgG2b followed by a horseradish peroxydase-streptavidin conjugate were used for detection of bound antibodies. Titers were calculated in relative to a standard mouse Ig and expressed as µg/ml.

4. Results

Experiment 1

Analysis of lymphoproliferation responses did not show any significant differences in reactivity to RTS,S between the groups. In contrast, the groups 1 and 3 containing both, CpG and DQS21, showed better gp120-specific Lymphoprolypheration responses than the groups containing CpG or DQS21 alone (Figure 6).

In this experiment only HBsAg-specific CTL were measured. There was no pronounced difference in CTL induction between the groups 1 and 3 having received CpG and DQS21 in combination and the groups 2 and 4 immunized with only one of the two adjuvant components, while the presence of $Al(OH)_3$ diminished the CTL activity observed for the combination of CpG and DQS21 in group 1(Figure 7). However, a trend was present that CpG and DQS21 was better than DQS21 alone, and the combination induced more CTL in the presence of $Al(OH)_3$ than CpG alone (Figure 7).

The humoral immune response of the mice was examined only for the presence of HBsAg-specific antibodies. Titers were similar in all groups expect for group 3, which showed an approximately three-fold increase, demonstrating that, in the presence of $Al(OH)_3$, the combination of DQS21 and CpG is more immunogenic than CpG alone (Figure 8). The isotype distribution was similar for the $Al(OH)_3$-containing groups 3 and 4, while in the absence of $Al(OH)_3$ the combination of CpG and DQS21 induced a stronger T_{H1}-like isotype pattern than DQS21 alone (Figure 8).

Experiment 2

Lymphoproliferation responses specific for RTS,S and gp120 were very similar in this experiment. The data indicate that the addition of DQS21 (either alone or with an o/w emulsion) enhances lymphoproliferation responses to both antigens (Figure 9).

CTL responses were evaluated by using both, an HBsAg and a gp120 CTL epitope peptide. In both cases, CTL could be detected after immunization of group 1 with CpG alone (Figure 10). However, addition of DQS21 resulted in a considerable increase in CTL for both antigens (Figure 10). The presence of an o/w emulsion either neutralized the positive effect of DQS21 (gp120) or increased the background of the in vitro assay (HBsAg).

Antibody responses to HBsAg and gp120 increased by addition of DQS21 to the CpG adjuvant (Figure 11A). A further increase was observed when an o/w emulsion was included in the formulation (Figure 11A). Addition of DQS21 to CpG shifted the gp120 isotype profiles towards a more pronounced T_{H1} bias (Figure 11B), while the impact on the HBsAg isotype profiles was less pronounced in this experiment.

5. Conclusions

Immunization with RTS,S and gp120 formulated with the combination of CpG and DQS21 results in strong antigen-specific immune responses. The combination of the adjuvant components CpG and DQS21

- enhances lymphoproliferation responses
- increases CTL activity
- augments antibody titers and T_{H1} isotype patterns

as compared to the single components.

EXAMPLE 5. ***Therapeutic potential of CpG and/or DQS21formulations in TC1 tumour model***

1. Experimental design

Four groups of 10 mice C57bl/6 received 10e6 (200 µl) TC1 cells (E7 expressing tumour cells) subcutaneous at day 0 in the flank.

Mice were then vaccinated twice at day 14 and 21 after the tumour challenge, with 5 µg of formulated PD1/3E7 HPV16 injected intra-footpad. Tumour growth was measured individually twice a week.

Groups of mice:

1. No vaccine
2. PD1/3E7 + CPG (10 µg ODN 2006)
3. PD1/3E7 + DQS21 (0.5 µg)
4. PD1/3 E7 + CPG + DQS21

The tumour growth was monitored by measuring individual tumours, twice a week.

2. Formulations

Formulations were performed the days of injections. The volume of injection for one mouse was 100 µl. When needed, PD1/3E7 (5 µg) was diluted with H_2O and PBS pH 7.4 for isotonicity. After 5 min., if needed QS21 (0.5 µg) mixed with liposomes in a weight ratio QS21/cholesterol of 1/5 (referred to as DQ) was added to the formulation. 30 min later, for the formulation with the oligo, 10 µg of CpG (ODN 2006) was added 30 min prior addition of 1 µg/ml of thiomersal as preservative.

H_2O + PBS pH 7.4 + PD1/3E7 - $_{5\ min}$ + DQ - $_{30\ min}$ + CpG - $_{30\ min}$ - Thio

3. Results

The evolution of the mean tumour growth per groups of 10 animals over time is shown in Figure 12. 100% of the animals that received a tumour challenge of 10e6 TC1 cells progressively developed growing tumour.

70-80% of the non vaccinated animals or of the animals vaccinated with the E7 protein in DQS21 died by day 35.

Two vaccinations with the E7 protein formulated in DQS21 had almost no effect on tumour growth. On the contrary, 2 vaccinations, IFP (day 14, 21) with 5 µg ProtD 1/3 E7 HPV16 in CPG adjuvant induced the regression of these pre-established tumours and protect mice from dying: 70- 80% of the mice were still alive at day 35. The combination of the 2 immunostimulants CPG and DQS21 showed a slight beneficial effect over the CpG used alone.

Claims

1. An adjuvant composition comprising a saponin and an immunostimulatory oligonucleotide.

2. An adjuvant composition according to claim 1 further comprising a carrier.

3. An adjuvant composition as claimed in claim 1 or 2, wherein said saponin is selected from the group comprising Quil A, or purified saponins such as QS21, QS7, QS17; β-escin, or digitonin.

4. An adjuvant composition as claimed in any one of claims 1 to 3, wherein said immunostimulatory oligonucleotide comprises a Purine, Purine, C, G, pyrimidine, pyrimidine sequence.

5. An adjuvant composition as claimed in claims 1 to 4, wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1); TCT CCC AGC GTG CGC CAT (SEQ ID NO:2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3); TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO:4); TCC ATG ACG TTC CTG ATG CT (SEQ ID NO:5).

6. An adjuvant composition according to claim 1 to 4, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated at least by 3 nucleotides.

7. An adjuvant composition according to claim 6, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated by 6 nucleotides.

8. An adjuvant composition as claimed in any one of claims 2 to 7, wherein said carrier is a particulate carrier selected from the group comprising mineral salts, emulsions, polymers, liposomes, ISCOMs.

9. A vaccine composition comprising an adjuvant composition as claimed in any one of claims 1 to 8, further comprising an antigen.

10. A vaccine composition as claimed in claim 9, wherein said antigen is derived from an organism selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus

type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Streptococcus, Mycoplasma, Mycobacteria, Haemophilus, Plasmodium or Toxoplasma, stanworth decapeptide; or Tumour associated antigens (TAA), MAGE, BAGE, GAGE, MUC-1, Her-2 neu, CEA, PSA, KSA, or PRAME; or a self peptide hormone, GnRH.

11. A vaccine composition as claimed in claim 9, wherein said antigen is derived from the group comprising (a) tumour associated antigens PSMA, PSCA, tyrosinase, survivin, NY-ESO1, prostase, PS108, RAGE, LAGE, HAGE; (b) or the N terminal 39 –43 amino acid fragment (Aβ) of the amyloid precursor protein; (c) or antigens associated to atherosclerosis.

12. A vaccine composition as claimed in claims 9 to 11 wherein the vaccine is administered systemically.

13. A vaccine composition as claimed in claims 9 to 11 wherein the vaccine is administered mucosally.

14. A vaccine composition as claimed in claim 13 wherein the saponin of the adjuvant composition is haemolytic.

15. A delivery device pre-filled with the vaccine of claims 9 to 11, said device being designed to administer the vaccine systemically.

16. A method of inducing an immune response in an individual, comprising the systemic administration of a safe and effective amount of the vaccine composition as claimed in claims 9 to 11.

17. A method of treatment of an individual susceptible to or suffering from a disease by the administration to an individual of an effective amount of the vaccine as claimed in any one of claims 9 to 14.

18. A method of treatment as claimed in claim 17, wherein the administration of the vaccine is through a systemic route.

19. A method of treatment of an individual suffering from a disease selected from the group comprising prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; non-cancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a vaccine as claimed in any one of claims 9 to 11.

20. A method for preventing an individual suffering from contracting a disease selected from the group comprising prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; non-cancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a vaccine as claimed in any one of claims 9 to 11.

21. A method of treatment as claimed in claims 19 and 20, wherein the vaccine is administered via a systemic route.

22. A vaccine as claimed in claim 9 or 11 for use as a medicament.

23. Use of a combination of a saponin and a CpG molecule in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer or other chronic disorders.

24. Use of combination of a saponin, an immunostimulatory oligonucleotide and a carrier in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer or other chronic disorders.

25. A method of inducing a systemic antigen specific immune response in a mammal, comprising administering to a mucosal surface of said mammal a composition comprising an antigen and a haemolytic saponin and a CpG molecule.

26. Method of making an adjuvant composition comprising admixing a saponin with an immunostimulatory oligonucleotide.

27. Method of making an adjuvant composition comprising admixing a saponin, an immunostimulatory oligonucleotide, and a carrier.

28. Method of making a vaccine comprising admixing the following (a) a saponin, (b) an immunostimulatory oligonucleotide, and (c) an antigen.

29. Method of making a vaccine comprising admixing the following (a) a saponin, (b) an immunostimulatory oligonucleotide, (c) a carrier and (d) an antigen.

Figure 1: serum IgG to lipo-OspA in mice

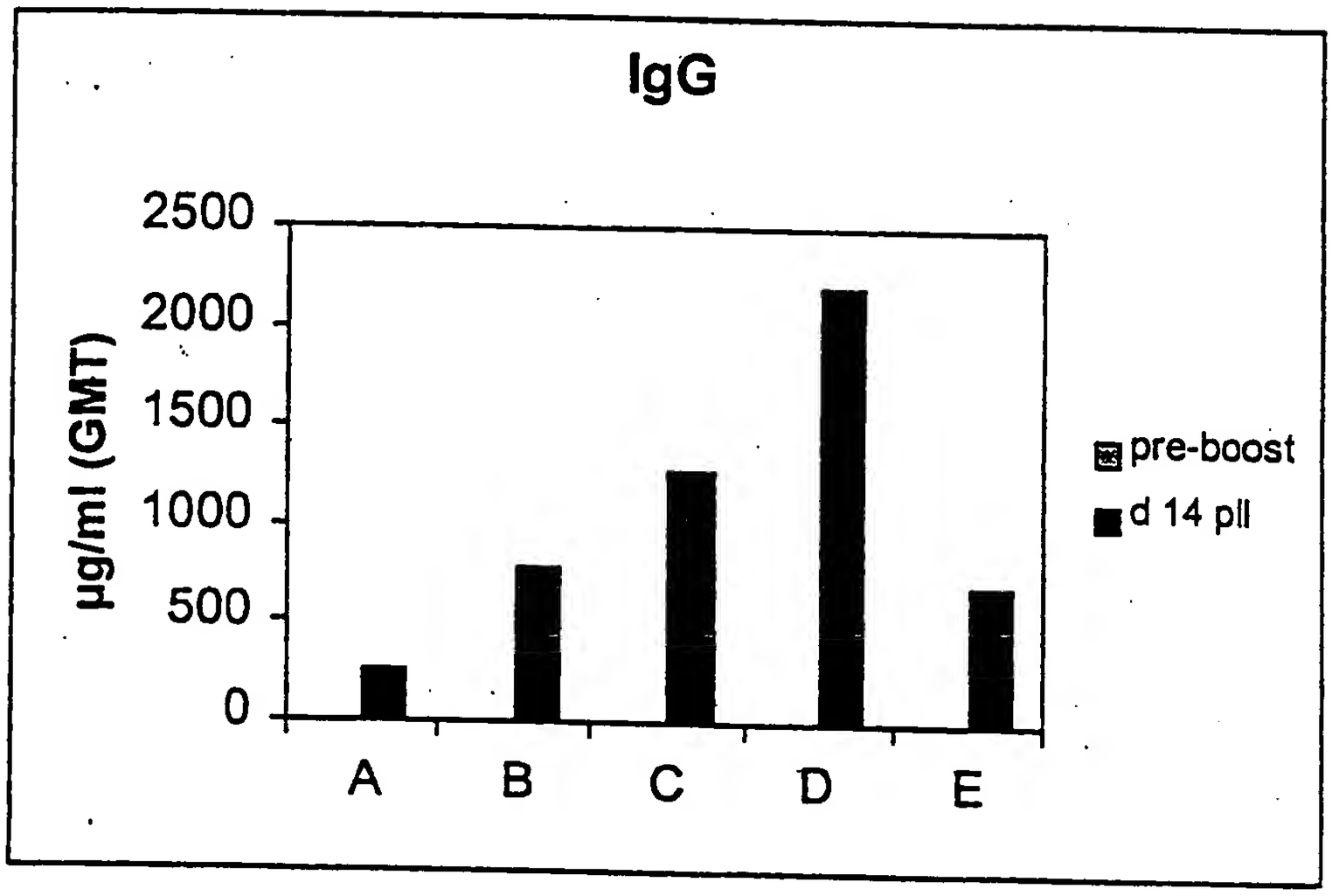


Figure 2: LA2 titres in mice

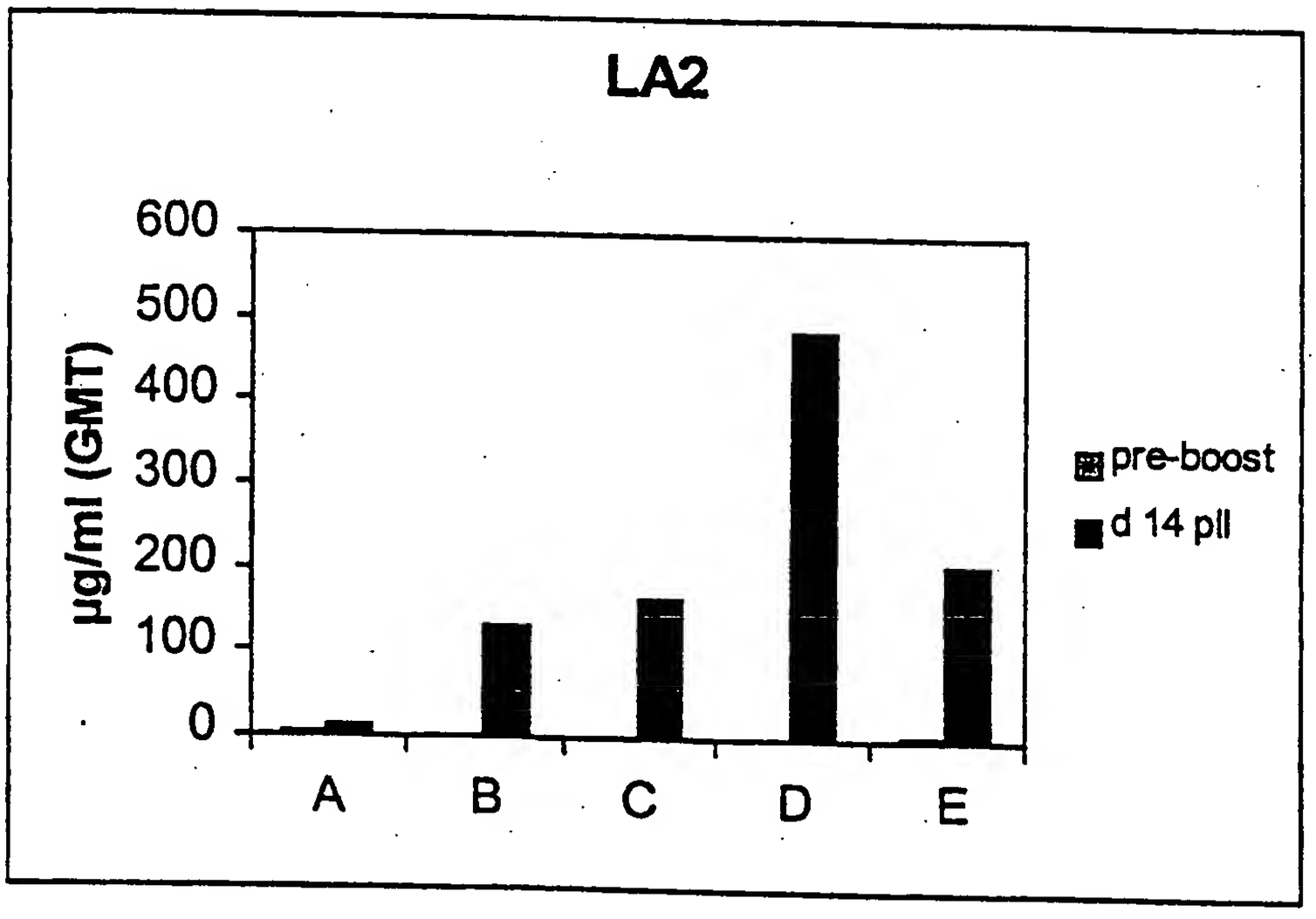


Figure 3: serum IgG titres to flu strains

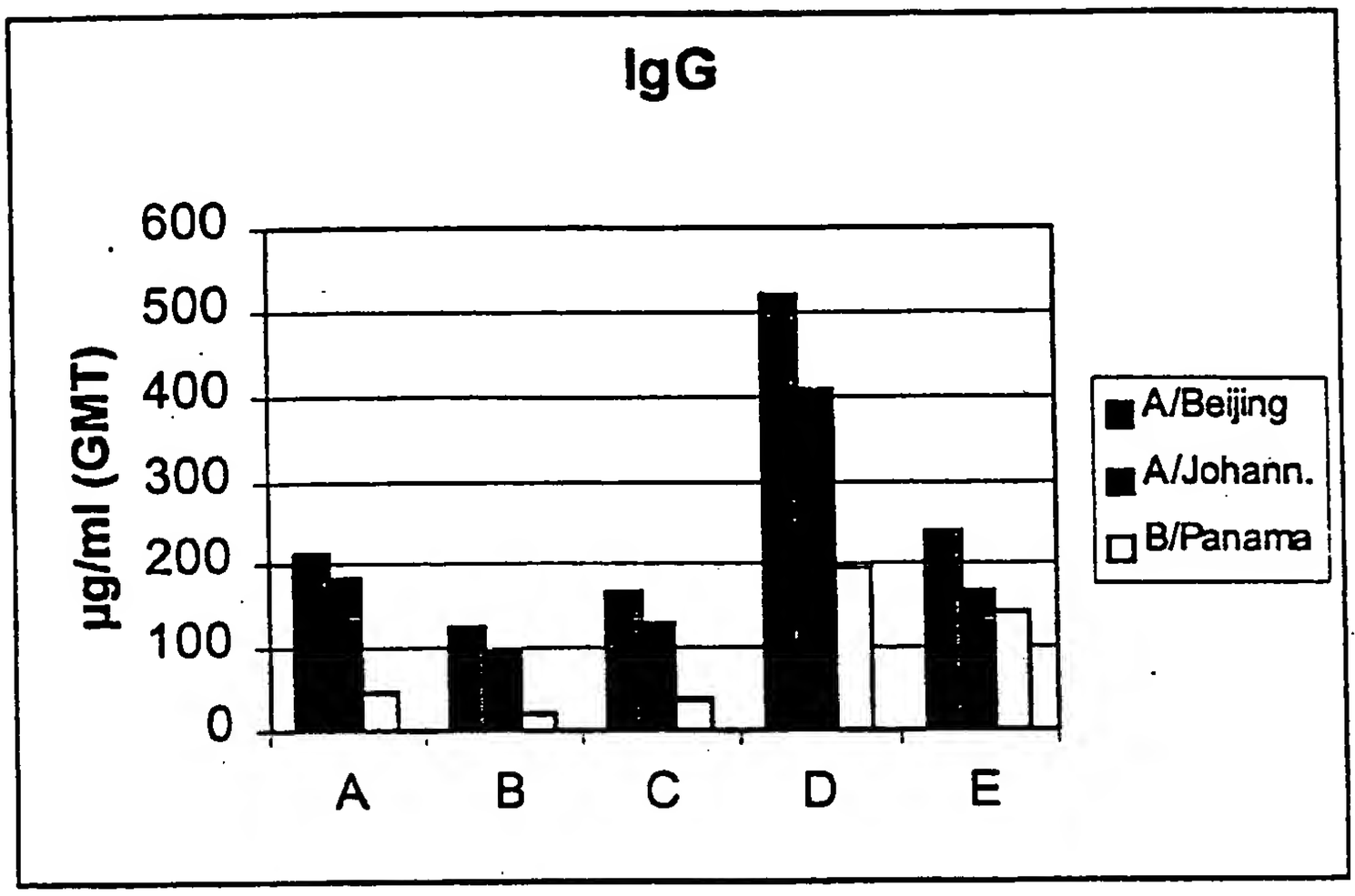


Figure 4: serum HAI titres

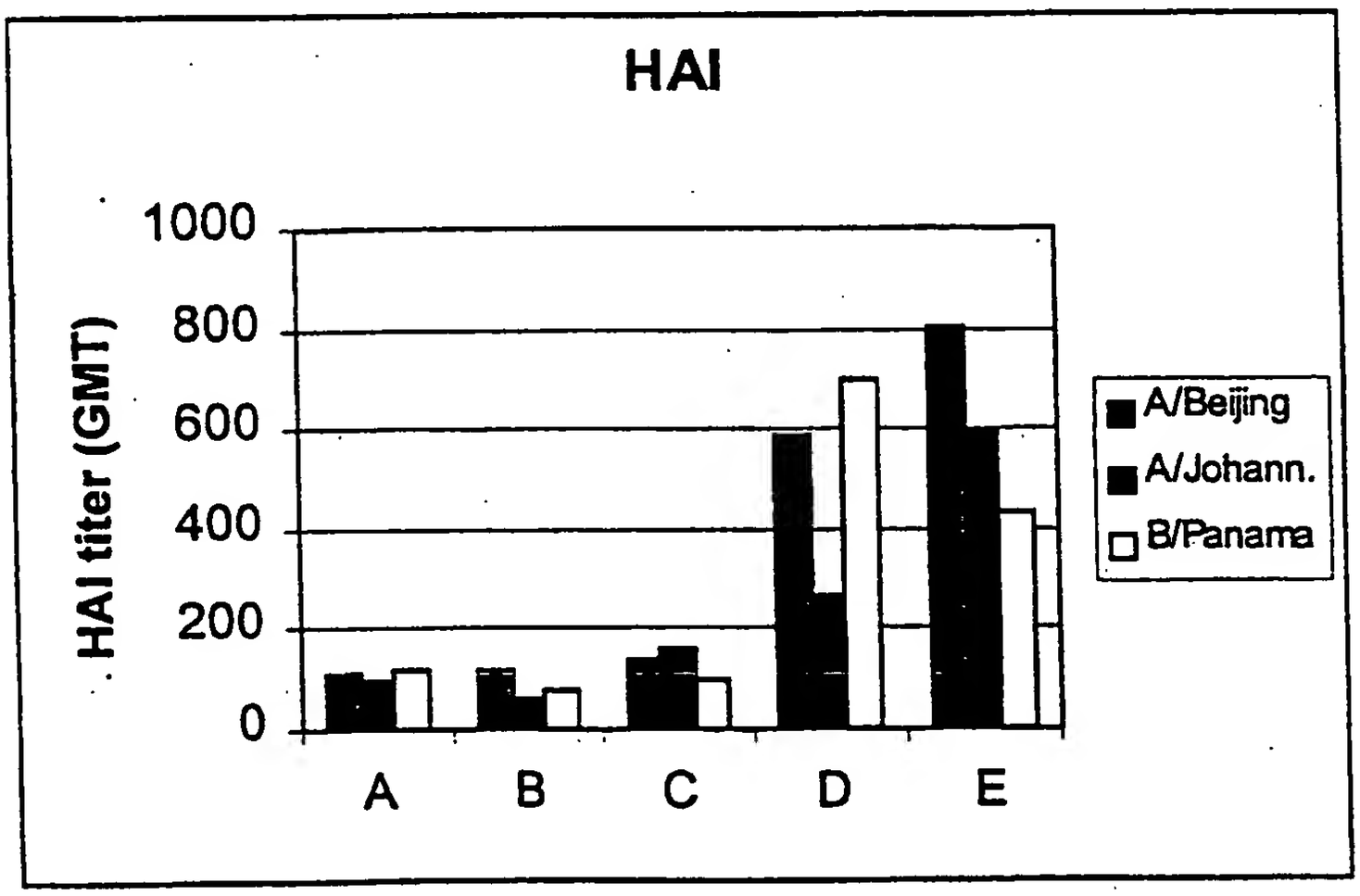


Figure 5, LA2 titres in mice

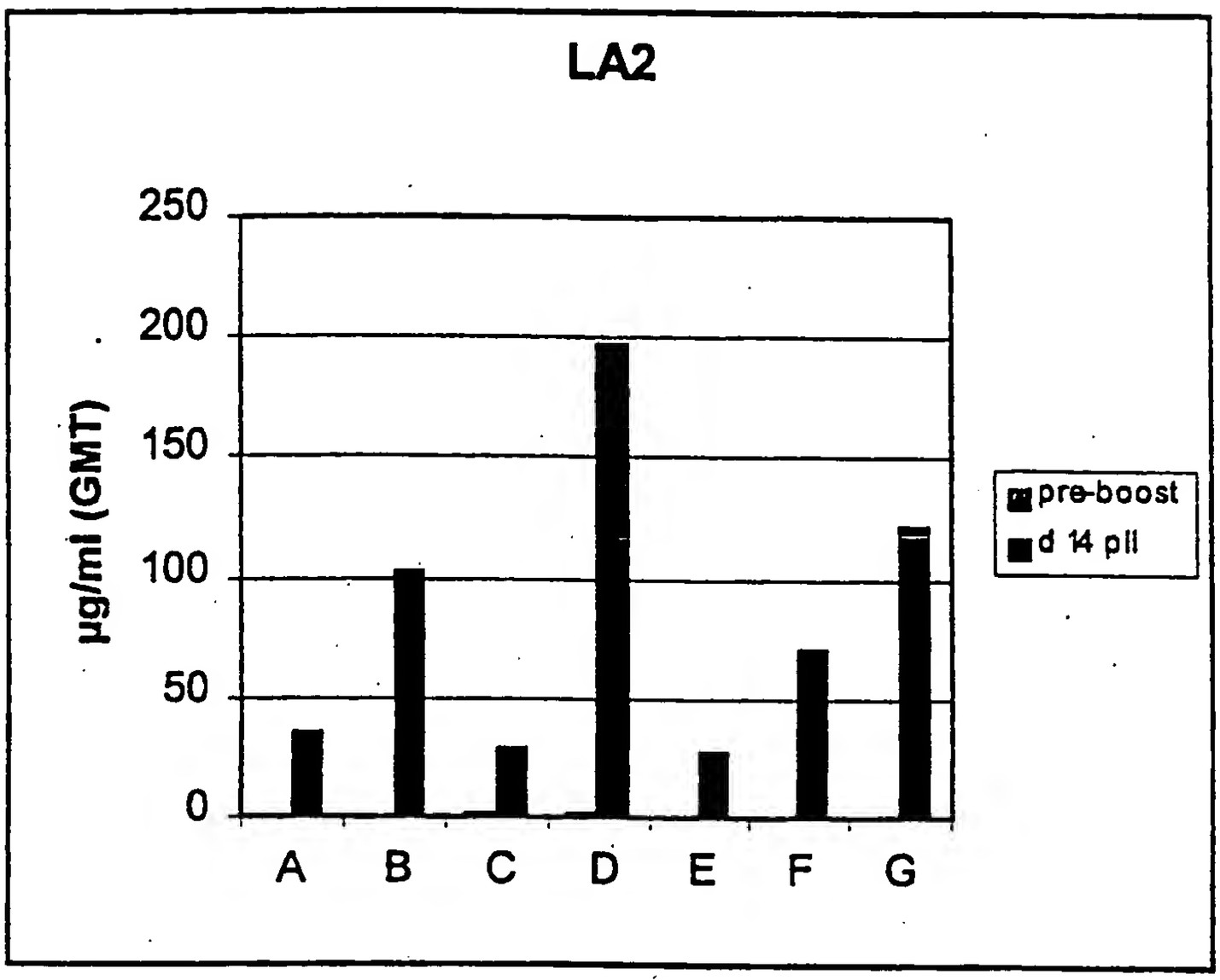


Figure 6: gp120-specific lymphoprolypherative activity of spleen cells from immunised mice.

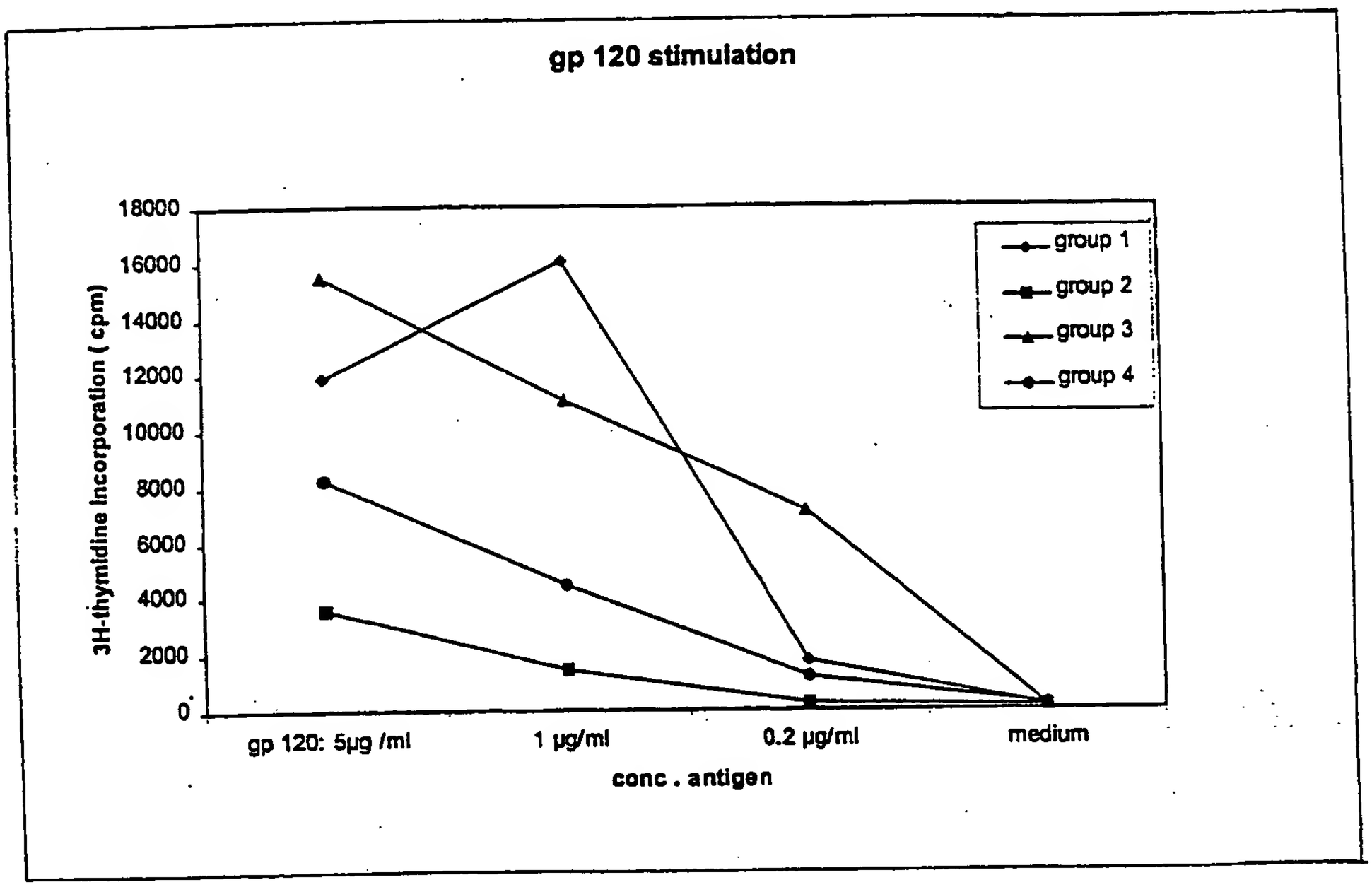


Figure 7: Stimulation with peptide HbsAg – CTL activity of spleen cells

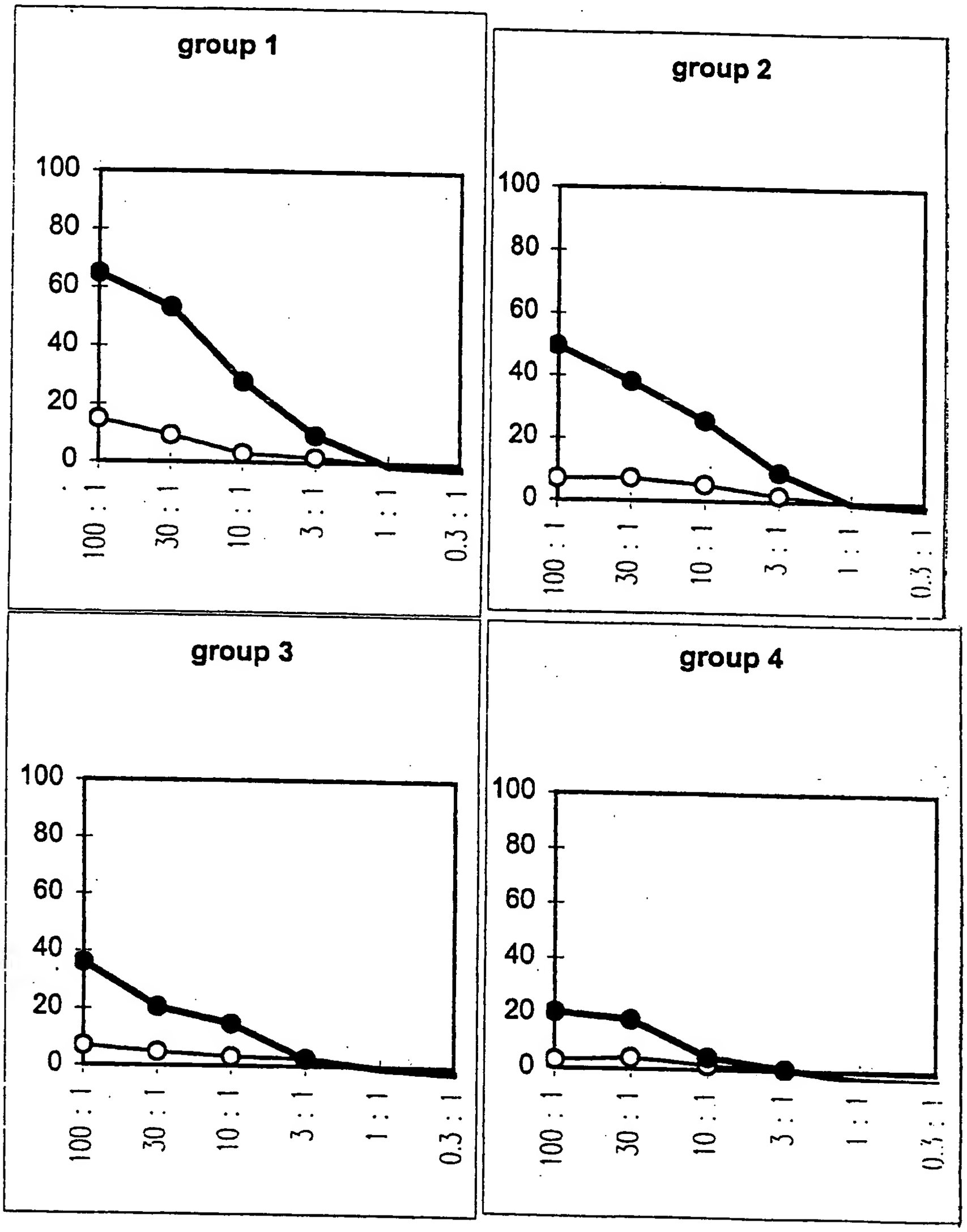
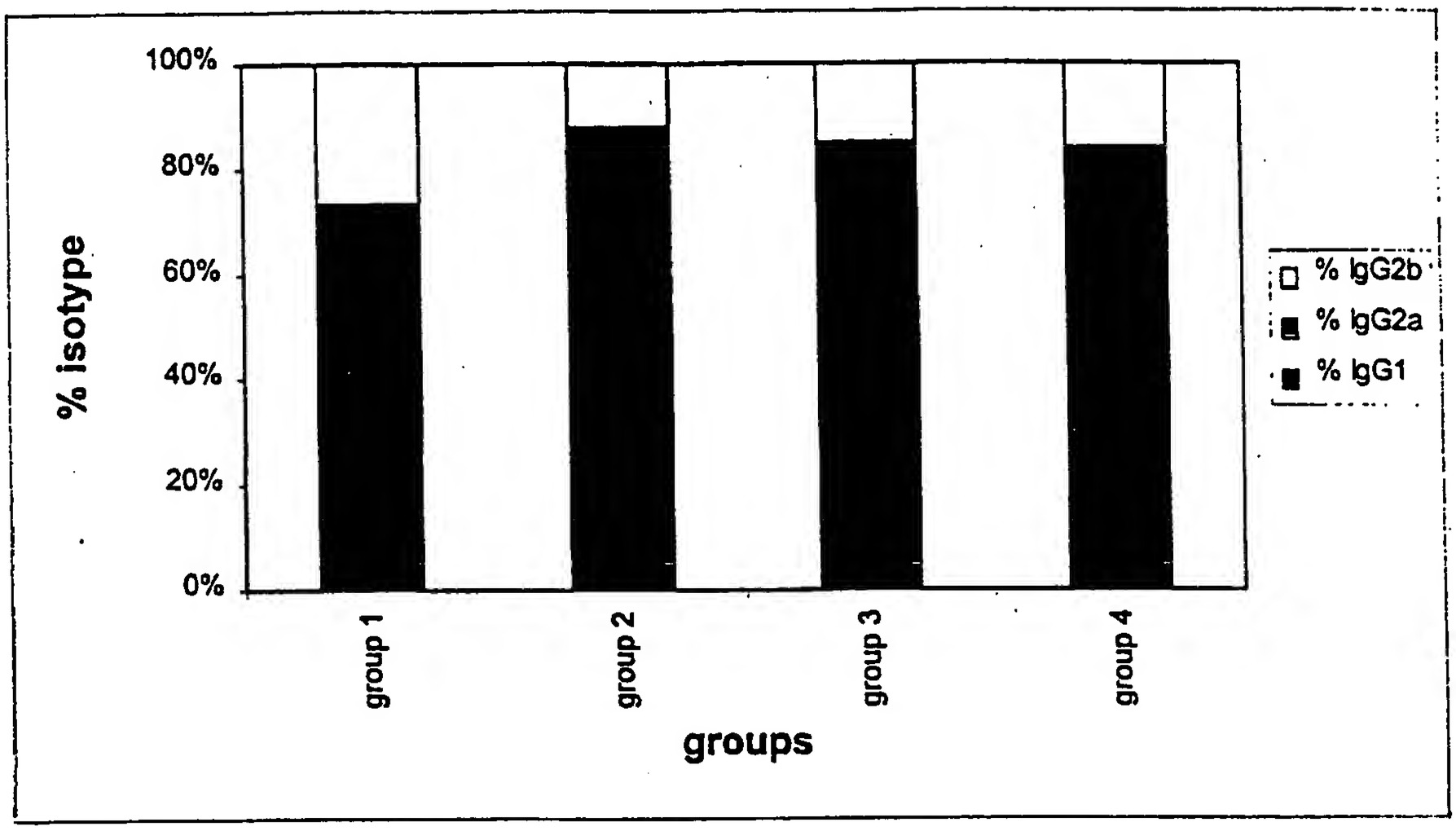


Figure 8: Isotype pattern of HbsAg-specific antibodies



HBsAg-specific antibody titers

formulations	Igtot UE/ml
group 1	31162
group 2	33689
group 3	95089
group 4	35690

Figure 9A: RTS,S stimulation

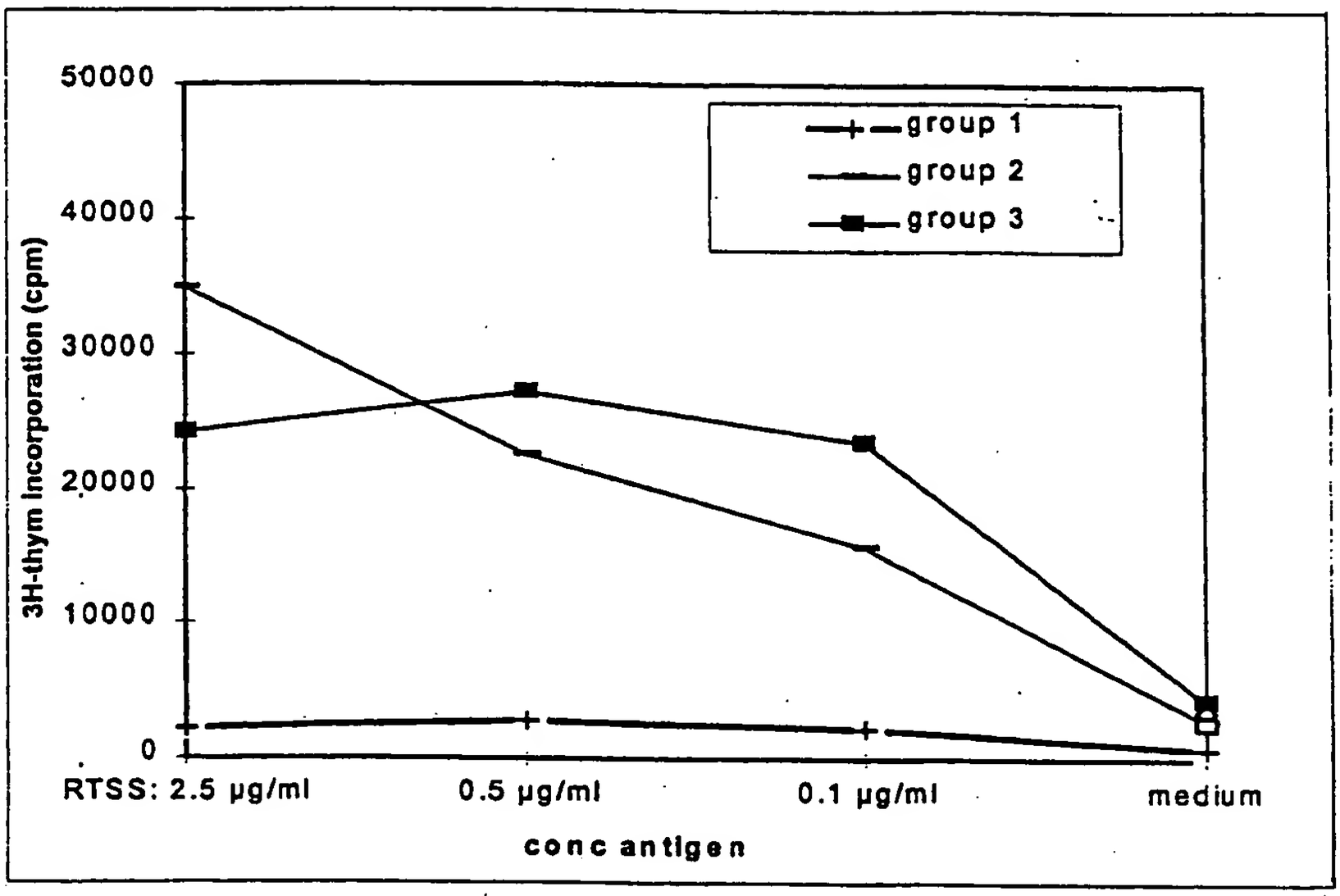
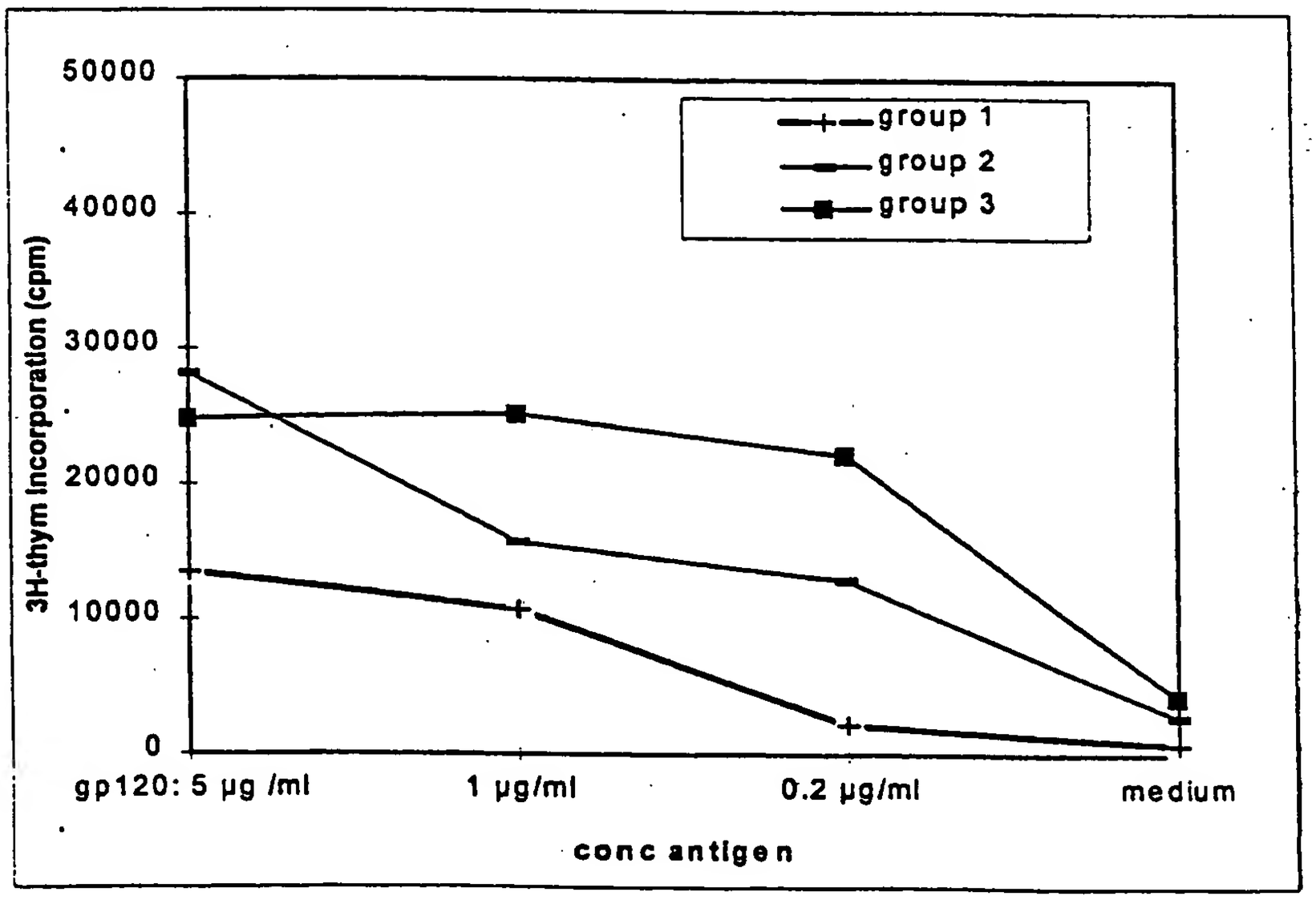


Figure 9B: gp120 stimulation



S

Figure 10A: Stimulation with peptide gp120

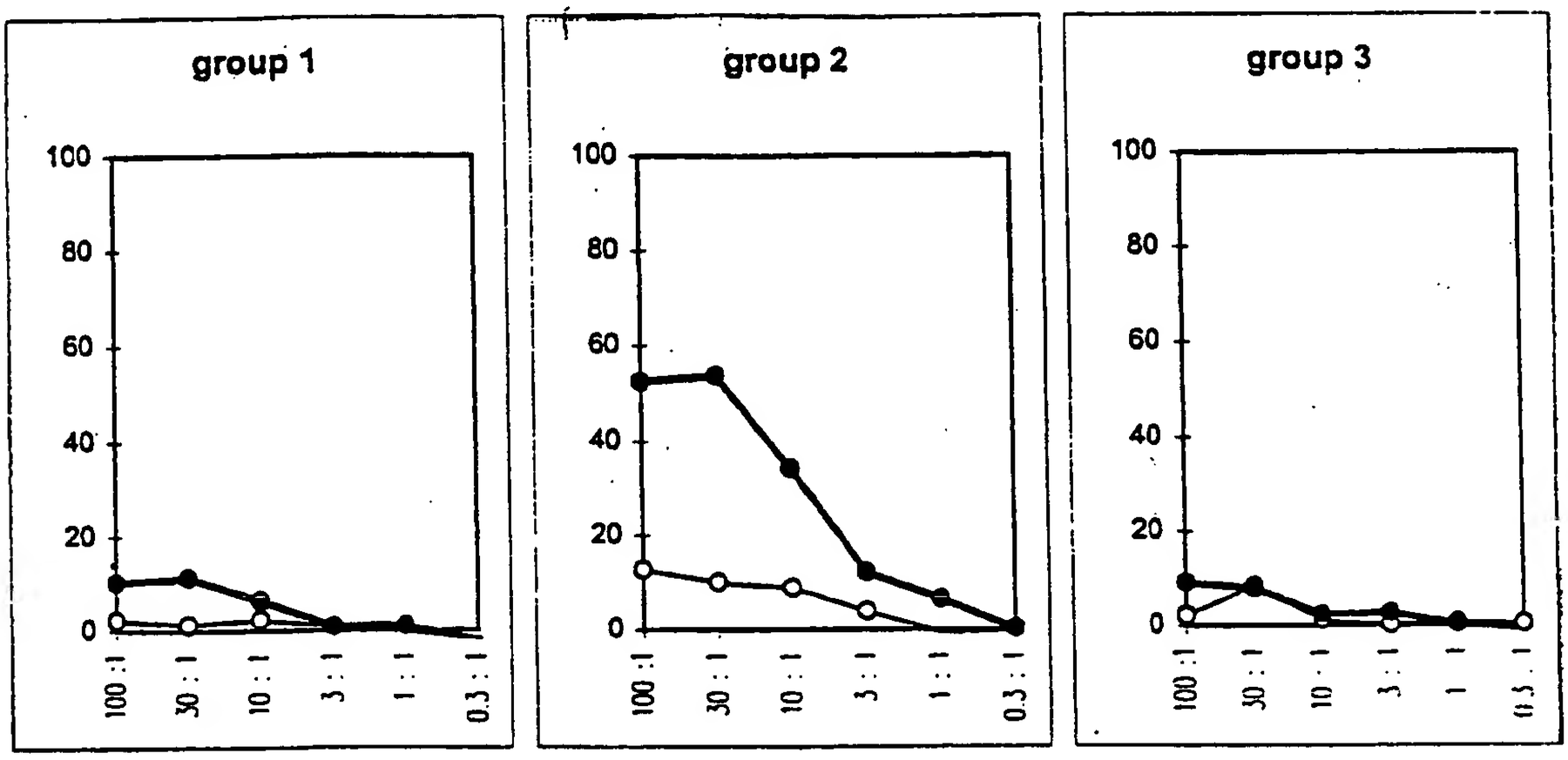


Figure 10B: Stimulation with peptide HbsAg

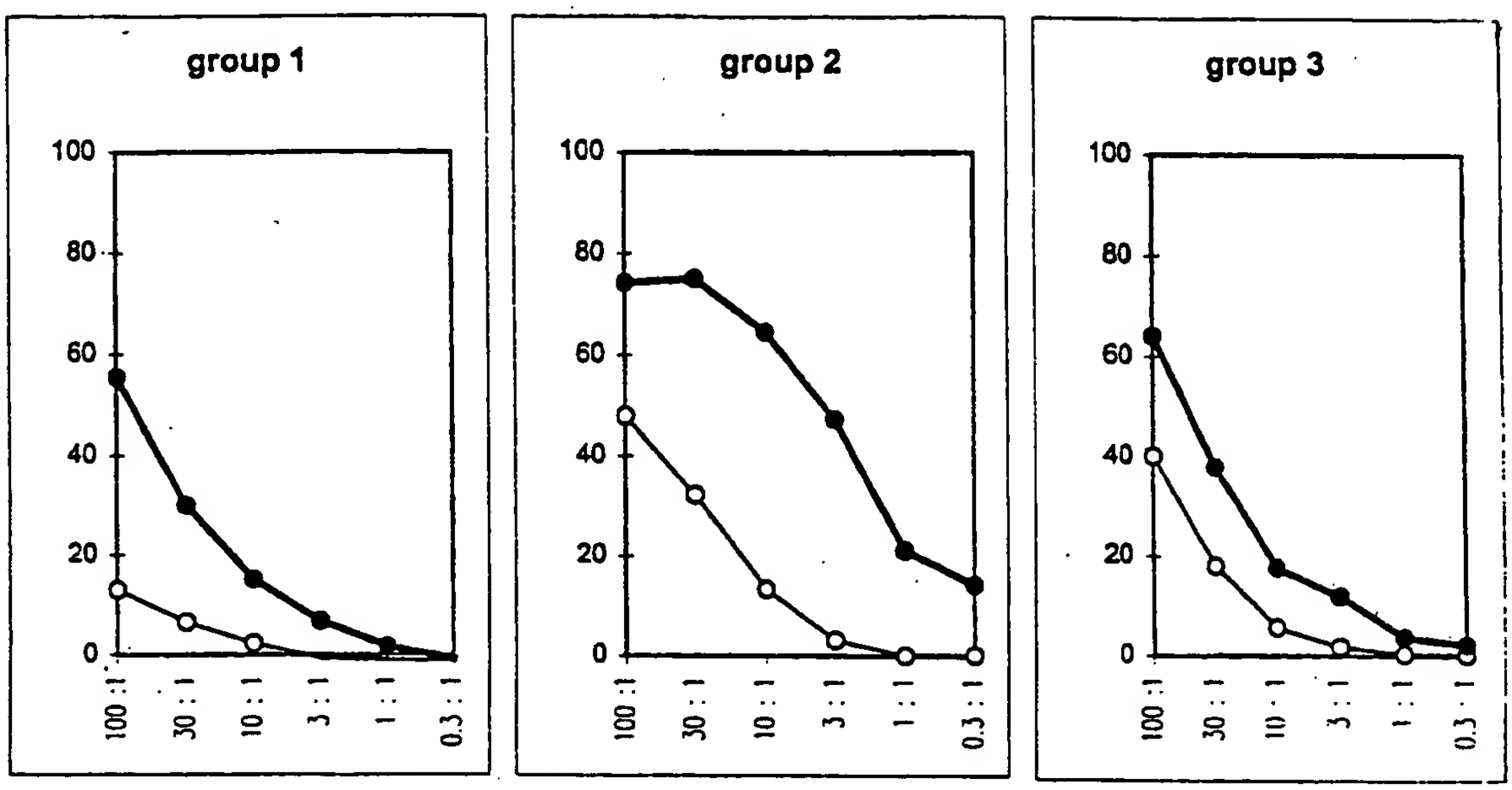


Figure 11A: HbsAg-specific and gp120-specific antibody titers

HBsAg-specific antibody titers

formulations	Igtot UE/ml
group 1	11536
group 2	30629
group 3	50540

gp120-specific antibody titers

formulations	Igtot µg/ml
group 1	24
group 2	62
group 3	92

Figure 11B: Isotype pattern of gp120-specific antibodies

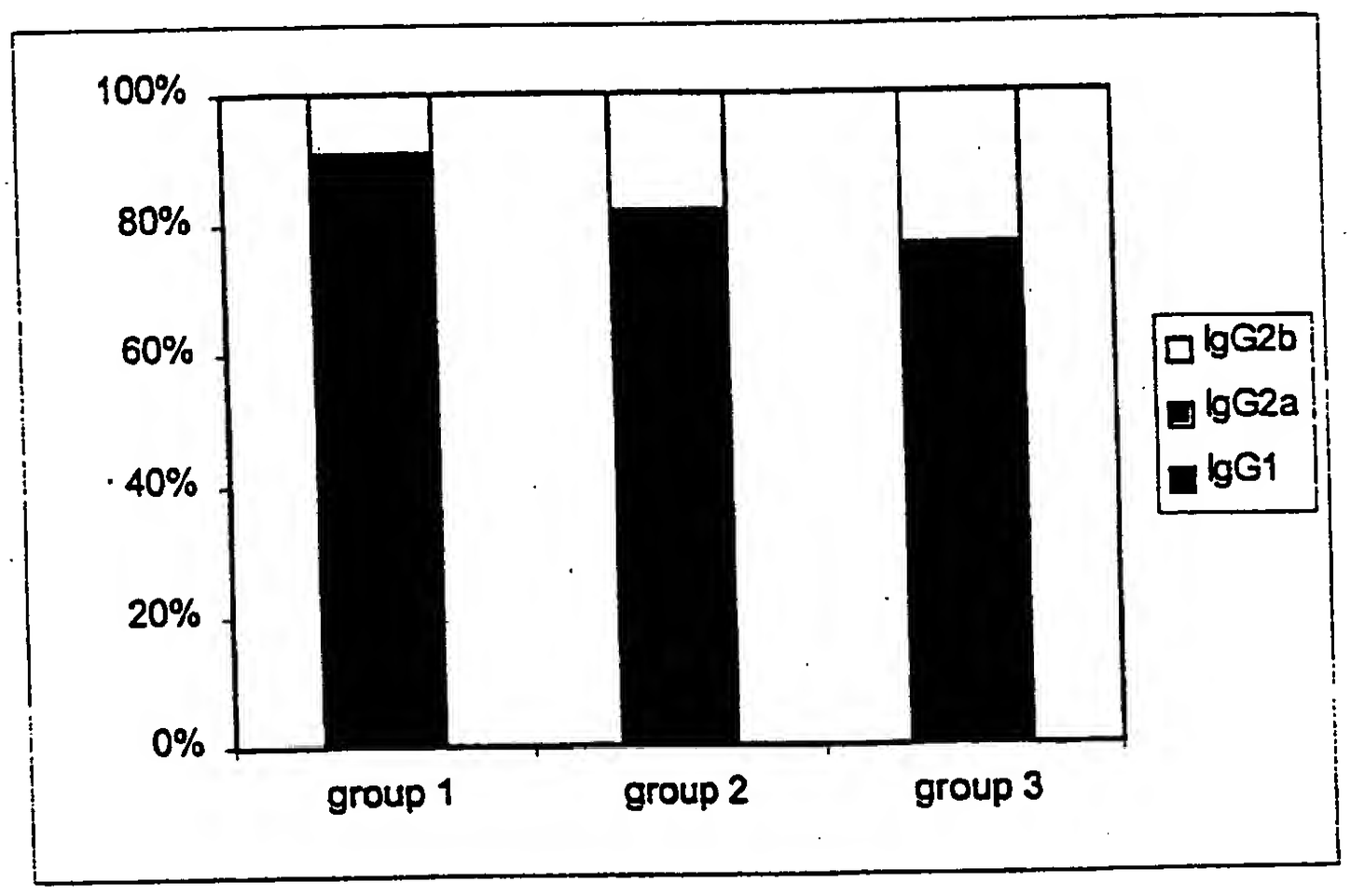
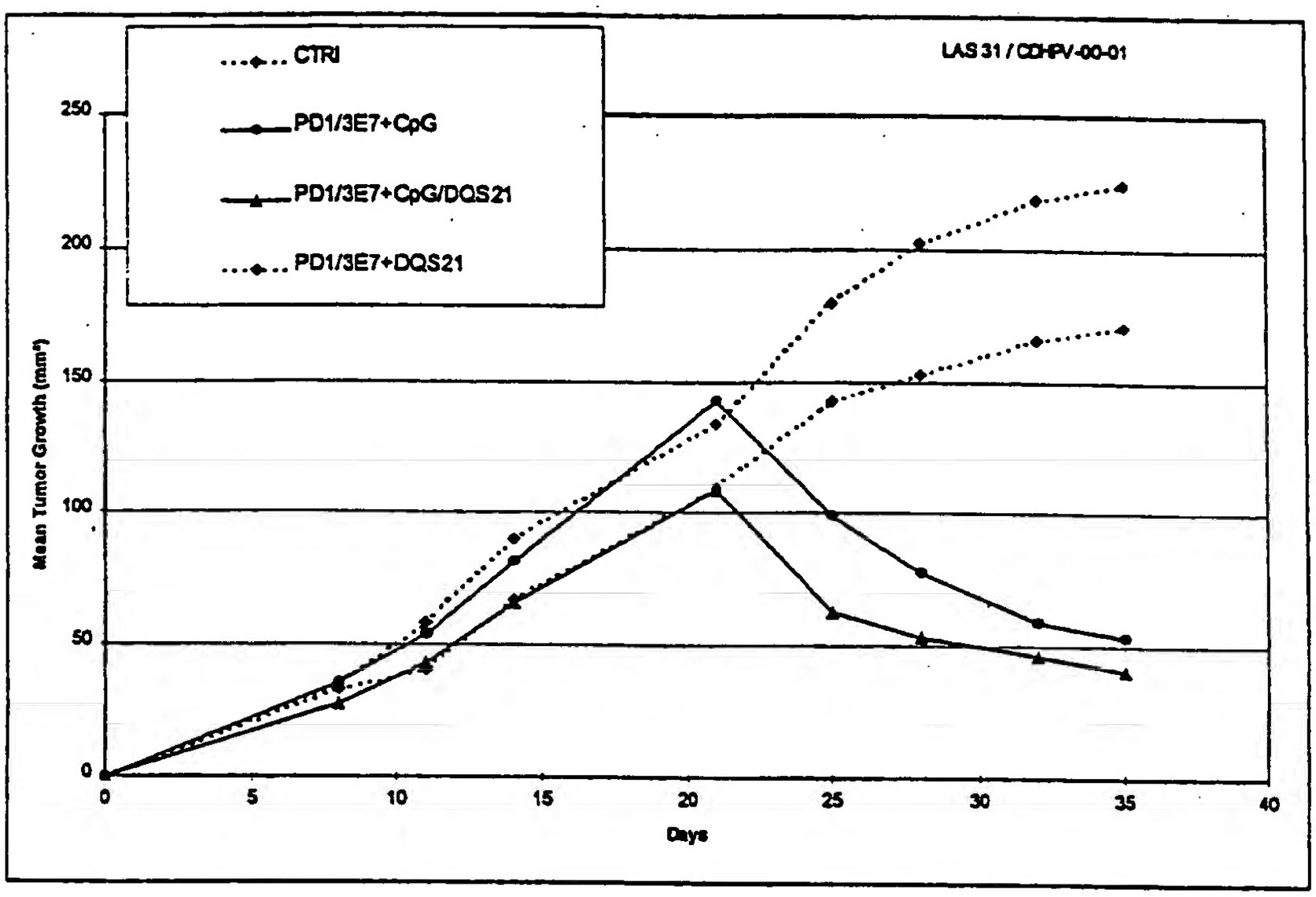


Figure 12: Tumour-growth per group of 10 animals over time



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Toll Meets Bacterial CpG-DNA

Minireview

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Discovery of *Drosophila* Toll and its eight homologs coupled with identification of homologous mammalian Toll-like receptors (TLRs) that discriminate "self" from pathogen-derived ligands (also termed pathogen associated molecular patterns [PAMPs]) (Hoffmann et al., 1999) has rejuvenated research on "innate immunity." Today we have come to realize that, for the recognition of pathogens, plants and insects have relied for millions of years upon a system of receptors that share a characteristic cytoplasmatic domain now termed TIR (Toll/interleukin-1 receptor domain). Amazingly, the TIR domain has remained conserved, and it functions in antipathogen responses in plants, insects, and mammalians alike. In mammals, 10 Toll homologs (TLRs) have been identified so far, all of which appear to be type I integral membrane proteins with extracellular leucine-rich repeats (LRRs) and cytoplasmatic TIRs (Hoffmann et al., 1999; Aderem and Ulevitch, 2000). It has also become clear that innate immune cells such as macrophages and dendritic cells (DCs) heavily impact on adaptive immune responses; innate immune cells control as antigen-presenting cells (APCs) whether T cells respond at all and whether emanating adaptive T cell responses become polarized toward Th1 or Th2 (Aderem and Ulevitch, 2000). Accordingly, we now argue that innate immunity was not only first but also effectively instructs subsequent adaptive responses to pathogens.

In flies, Toll is involved in antifungal responses, and one of its homologs, 18-wheeler, confers responsiveness to Gram-negative bacteria (Hoffmann et al., 1999). In a seminal report, Janeway and colleagues identified the first human homolog of *Drosophila* Toll (Medzhitov et al., 1997), and subsequent positional cloning revealed that it encodes a signal transduction and receptor component specific for lipopolysaccharide (LPS) (Poltorak et al., 1998). In vitro genetic complementation of nonresponder cells with TLRs and gene knockout studies revealed that TLR2 acts as the essential receptor component for a variety of PAMPs, including lipopeptides from bacteria and mycobacteria, peptidoglycans, phenol-soluble modulin from Gram-positive bacteria and whole Gram-positive bacteria, as well as zymosan from yeast (Aderem and Ulevitch, 2000). At first view, the broad spectrum of receptor properties of TLR2 contrasts the concept of ligand-specific PAMP-TLR interactions. However, in equivalent assays at least several hundred-fold higher concentrations of, for example, peptidoglycan are required when compared to bacterial lipopeptides, thus raising the question of potential contaminations. Furthermore, recent evidence of cooperation between TLRs in specific PAMP recognition may also help overcome this concern. For example, it has been suggested that TLR2 forms functional pairs with TLR6 and TLR1, respectively, implying the potential of a combinatorial repertoire generated by heterodimerization of TLRs (Hajjar et al., 2001; Ozinsky et al., 2000) (Figure 1). In contrast, the hallmarks of adaptive immunity include the principle of somatic recombination to generate the diverse T cell and B cell receptor repertoire and the ability to form memory responses upon pathogen exposure.

TLR-Initiated Signaling

As far as it is known, all TLRs activate innate immune cells via the Toll/interleukin-1 receptor signal pathway (Hoffmann et al., 1999; Aderem and Ulevitch, 2000) (see Figure 1). As a first cytosolic event, the adaptor molecule MyD88 is recruited to the receptor complex, followed by engagement of IL-1 receptor associated kinases (IRAKs) and the adaptor molecule TRAF6. Oligomerization of TRAF6 leads to activation of downstream kinases like the stress kinases c-Jun N-terminal kinase (JNK), p38, and IκB kinase (IKK) complex. This results in the activation of transcription factors like AP-1 and NF-κB. Recruitment of MyD88 seems to be an essential step since MyD88-deficient cells exhibit severe impairment of innate immune responses to PAMPs. Interestingly, upon phagocytosis of particles including living bacteria, TLRs become recruited to phagosomes, presumably to screen the contents for their ligands and subsequently to trigger signaling via MyD88 (Ozinsky et al., 2000).

The idea that PAMPs activated TLRs uniformly and exclusively initiate signaling via the TLR/IL-1 receptor pathway may turn out to be too simplistic. For example, the cytoplasmatic TIRs of distinct TLRs appear not to be functionally equivalent, since TLR1, TLR2, and TLR6 are believed to require heterodimenerization to induce TNF-α production in macrophages, while TLR4 functions on its own (Hajjar et al., 2001; Ozinsky et al., 2000). Interestingly, TLR2 appears to activate a signaling cascade composed of the Rho GTPase Rac1, the phosphatidylinositol-3 kinase (PI3K), and Akt that targets nuclear NF-κB transactivation independently of IκBα degradation (Arbibe et al., 2000). In addition, MyD88 appears to couple TLR2 to the Fas associated death domain protein (FADD)/caspase 8 apoptotic machinery (Aliprantis et al., 2000), and signaling via the newly defined TIR associated protein (TIRap) reportedly bifurcates upstream of MyD88 at the level of TIR (R. Medzhitov; Conference on Innate Immunity, Keystone 2001). Furthermore, nucleosome remodeling of chromatin structure at the IL-12 p40 promoter in macrophages has been described as one of the endpoints in endotoxin—TLR4 triggered TIR domain signal transduction, an endpoint that is independent of NF-κB activation (Weinmann et al., 2001). Finally, the innate immune system may have evolved to express different TLRs (or combinations thereof) within different subsets of immune cells. For example, human CD123$^+$

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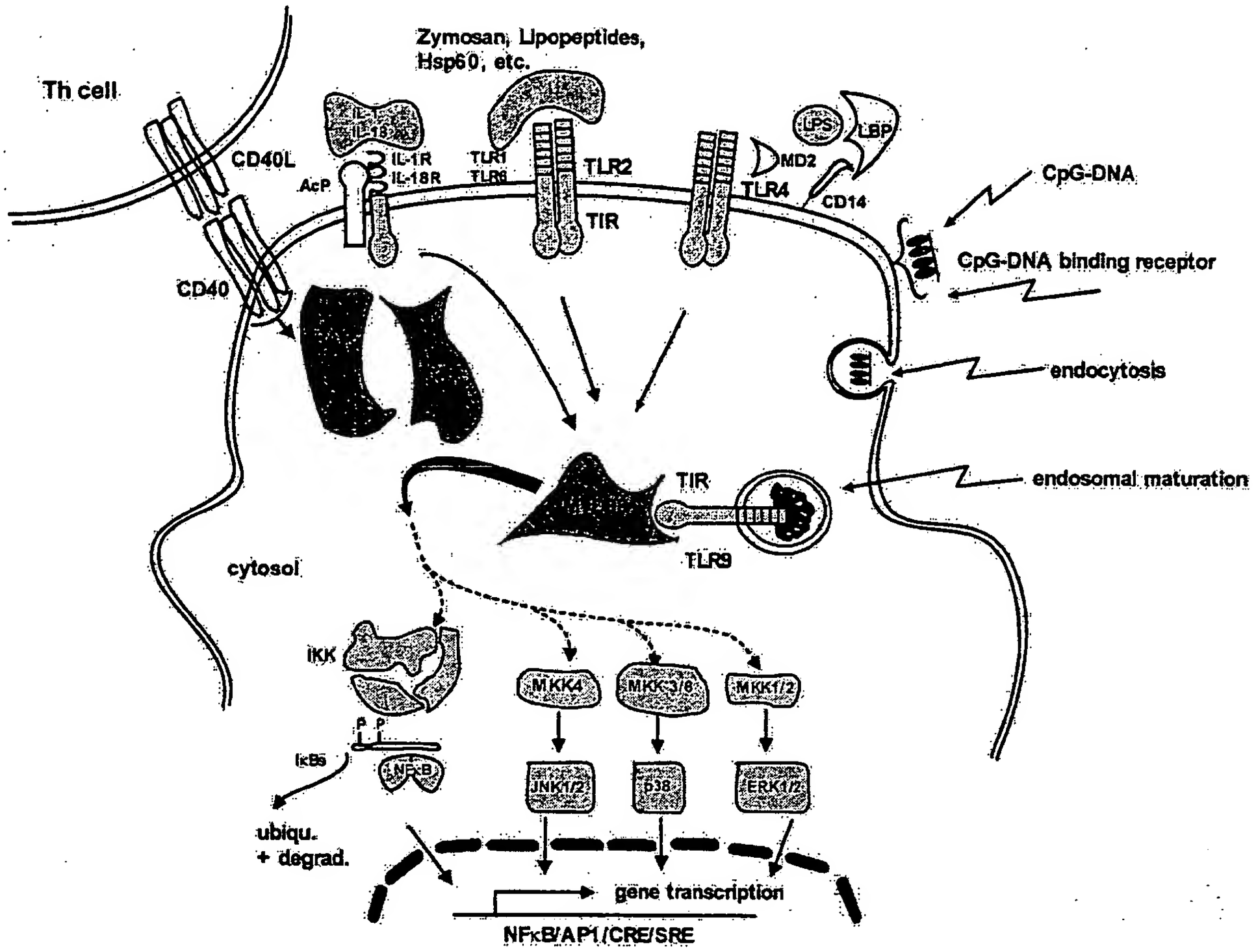

Figure 1. Signaling Pathway Activated by TLR9

Bacterial CpG-DNA or mimicking CpG-ODN first binds to a cell surface DNA binding protein. Binding is sequence independent and does not bring about cell activation but rather endocytosis. Upon endosomal maturation/acidification, immunostimulatory CpG motifs become sequence-specifically engaged by TLR9. Activated TLR9 recruits the adaptor protein MyD88 followed by IRAK and TRAF6. Ligands for IL-1R, IL-18R, TLR2, and TLR4 essentially trigger a similar signal cascade, while CD40L expressing T helper cells interact at the level of TRAF6. Arguments supporting the involvement of MyD88, TRAF6, Iκ kinase α/β, and TLR9 are exclusively based on experiments using dominant-negative forms of the respective proteins, genetic complementation with TLRs of nonresponder cells, and analysis of MyD88- or TLR9-deficient mice. The glycosyl-phosphatilinositol linked CD14 molecule lacks a signal transduction moiety of its own and is thought to scavenge LPS opsonized by LPS binding protein (LBP) for recognition by TLR4, and assisted by MD2. All molecules in the figure are symbols only—their shapes are not based on biochemical data. Solid arrows indicate direct interactions. Broken arrows are used where direct interactions has not been demonstrated.

$CD11c^-$ lymphoid precursors of DCs respond to CpG-DNA but not to LPS, while $CD11c^+CD123^-$ myeloid precursors are responsive to LPS but not to CpG-DNA. Since the former primarily produce type I interferons and the latter IL-12, the specificity of the immune responses appears to depend on the effector cells that became activated.

Bacterial CpG-DNA and TLR9

Bacterial genomic CpG-DNA and mimicking CpG-oligonucleotides (ODN) represent the most recent example of the power of pathogen-derived PAMPs to influence "adaptive" immune responses by activating "innate" immune cells such as macrophages and DCs (Wagner, 1999). In 1984, while attempting to identify the antitumor components of bacillus Calmette-Guerin (BCG) extracts, Tokunaga and colleagues first established that it might be bacterial genomic DNA alone that could account for the observed immune stimulation and antitumor effects. In a seminal report (Krieg et al., 1995), it was subsequently reported that mitogenic activity of bacterial DNA to murine B cells was due to the presence of unmethylated CpG dinucleotides in a particular base sequence context termed "CpG motif." Importantly, the immunostimulatory activity is found in bacterial, yeast, insect, and nematode DNA, while mammalian cells ignore even high concentrations of their own DNA. Furthermore, bacterial CpG-DNA or mimicking CpG-ODN activate macrophages and DCs in vitro and in vivo to produce proinflammatory cytokines such as TNF-α, IL-1, and IL-6, as well as effector cytokines such as IL-12 and IL-15. In addition, CpG-DNA causes immature antigen-presenting cells (APCs) to mature to professional (licensed) APCs able to activate antigen-reactive naive T cells. In mice, the potent innate immune cell activation

by CpG-DNA is sufficient to protect and even to treat established infections by intracellular pathogens including that of *Leishmania* major. Most likely, CpG-DNA drive adaptive immunity via activation of DCs that provide a Th1 polarizing environment in the secondary lymphoid organs where antigen-reactive T cells are exposed to licensed APCs in the presence of increased levels of cytokines such as IL-12. Indeed, it has become clear that CpG-DNA is a potent Th1-like adjuvant matching the potency of complete Freunds, which not only promotes "cross-priming" of MHC class I restricted cytolytic T cell (CTL) responses to peptides and proteins but also triggers Th1-polarized robust antibody responses. Remarkably, the strength of CpG-DNA as an adjuvant renders CTL responses toward proteins and peptides independent of T cell help (Cho et al., 2000; Krieg and Wagner, 2000).

The sequence specificity of CpG-DNA-driven responses is best documented by the fact that reversal of cytosine-guanosine (CG)-dinucleotides to GC or methylation of C is sufficient to ablate its immunostimulatory activity. Such a clear structure-function relationship implies the existence of a receptor with specificity for the CpG motif. Building on functional parallels between LPS and CpG-DNA-driven innate immune cell activation, subsequent studies (Hacker et al., 1998) revealed that in response to CpG-DNA challenge, classical signaling events are switched on such as the stress kinase pathway and the NF-κB pathway, as is the case for LPS. Since APCs stimulated by LPS or CD40 ligand expressing T helper cells become activated via MyD88 or TRAF6, respectively, we tested whether CpG-DNA also activates the Toll/IL-1 receptor signal pathway. In collaboration with S. Akira's group, it could be shown that inactivation of either MyD88 or TRAF6 abolished CpG-DNA signaling (Hacker et al., 2000). These results suggested a potential role of TLRs as CpG-DNA receptor. Indeed, macrophages and DCs of TLR9-deficient mice turned out to be selectively nonresponsive to CpG-DNA (Hemmi et al., 2000). In addition, genetic complementation of human nonresponder cells with either human or murine TLR9 restored responsiveness to bacterial DNA and CpG-ODN in a species-specific manner (Bauer et al., 2001). For example, complementation with human TLR9 conferred responsiveness to "optimal" human CpG motifs, while complementation with murine TLR9 conferred responsiveness to murine CpG motifs. Overall, these data imply that TLR9 conveys CpG-DNA responsiveness to cells by directly engaging immunostimulating CpG-DNA. In addition, the extracellular region of TLR9 contains a potential DNA binding motif, perturbation of which destroys CpG-DNA-driven responses.

Consistent with published evidence that endocytosis of CpG-DNA is required for its immunostimulatory activity, CpG-DNA-stimulated macrophages recruit MyD88 to endosome-like vesicular structures where it colocalized with CpG-DNA and thus initiates signaling. In contrast, LPS does not require endocytosis for signaling and MyD88 is recruited to the cell membrane. Altogether, these results support the conclusion that TLR9 sequence specifically recognizes CpG-DNA to initiate signaling by recruiting MyD88.

Independently, E. Raz and colleagues (Chu et al., 2000) have reported that DNA-dependent protein kinase (DNA-PK) is required for CpG-DNA recognition by innate immune cells. DNA-PK, a member of the PI3 kinase family, is known to be activated following recognition of double-stranded DNA breaks and single-stranded DNA ends, yet the implication of sequence-specific DNA recognition is novel. Furthermore, the data imply that CpG-DNA activated cytoplasmatic DNA-PK directly phosphorilate IκB kinase β, thus liberating NF-κB in a MyD88-independent fashion. At present, a connection between TLR9 and DNA-PK as receptors for CpG-DNA is not immediately apparent, since SCID mice known to carry an inactivating mutation in the DNA-PK gene perfectly respond to CpG-DNA (Wagner, 1999). It will be important to determine whether TLR9 or DNA-PK sequence specifically binds and thus recognizes CpG-DNA.

Questions on TLR9 and CpG Motifs

Shuffling of protein effector domains and effector motifs to create functionally new proteins appears to be a feature in evolution of higher organisms. This raises the question of how and when in evolution the TIR domain became connected with LRRs that harbor a DNA binding domain allowing sequence-specific recognition of bacterial CpG-DNA. Integration of foreign bacterial or viral DNA is considered as genotoxic by causing genomic instability of host cells. Methylation of bacterial and viral CpG-DNA has thus been viewed as an ancient defense mechanism to inactivate invaders DNA. To silence genes, most but not all CpG dinucleotides are methylated in genomic vertebrate DNA. Yet, methylated CpG dinucleotides represent mutational hot spots due to spontaneous deamination of 5-methyl-cytosines. As a consequence, methylated CpGs tend to be lost from genomes of vertebrates, a phenomenon termed CpG suppression. One may predict that evolution of structural differences between vertebrate and bacterial DNA such as frequency and methylation of CpG dinucleotides preceded its use as invariant microbial molecular pattern to be recognized by TLR9. However, upon demethylation mammalian genomic DNA remains immunologically inert, implying that not only the frequency of CG-dinucleotides but also that of immunostimulatory CpG motifs is suppressed. This assumption would explain why unmethylated CpG-dinucleotides that are frequent in mammalian promoters have no immunostimulating potential. Perhaps it is the presence of inhibitory sequences (Krieg et al., 1998) within vertebrate DNA that compete with stimulatory CpG motifs in activating innate immune cells. Alternatively, perhaps there has to be certain density of stimulatory motifs within a given DNA sequence in order for TLR9 to act. It is, however, hard to conceive that innate immune cells could impact so heavily on the DNA composition of the organism. The evolutionary origin of CpG-DNA recognition by TLR9 therefore remains mysterious.

Eye Outward or Inward

TLRs can be viewed as eyes of innate immune cells turned outward to identify conserved molecular patterns of pathogens and danger signals originating from stressed or injured cells. For example, human recombinant heat shock protein (hsp)60 triggers in macrophages the Toll/IL-1 receptor signal pathway via MyD88 and TRAF6 by engaging either TLR2 or TLR4 (unpublished data).

It would be, however, detrimental if epithelial cells, lining the colon and thus bathing in bacteria and bacte-

rial products, were responsive to these "inflammatory" agents. Indeed, epithelial cells are refractory to "exogenous" LPS. Nevertheless, certain pathogens such as invasive *Shigella flexneri* or microinjected endotoxin appear to activate NF-κB through an LPS-driven intracellular response that leads to IL-8 expression; noninvasive *S. flexneri* fail to do so (Philpott et al., 2000). Although these data could reflect differences in LPS presentation to TLR4, they also could give a hint of the existence of cytoplasmatic recognition structures capable of detecting intracellular PAMPs. Candidates include the recently described Nod1 and Nod2 proteins, since genetic complementation of nonresponder cells with Nod1 or Nod2 conferred responsiveness to LPS in a TLR4- and MyD88-independent manner. The leucine-rich repeats of Nod1 and Nod2 were required for LPS-induced NF-κB activation, and LPS binding activity could specifically be immunopurified with Nod1 from cytosolic extracts (Inohara et al., 2000). Although other interpretations may exist, it is proposed that TLRs screen "outward" for ligands of either the microbial world or hsps released after cell injury, while Nod family members are turned "inward" to screen the cytoplasm for PAMPs derived from invasive bacteria.

Concluding Remarks

In the context of disease, the term "immunity" can be traced back to the 14th century to describe resistance to plague. The term was thus available in 1883, when E. Metchnikov dared to suggest that phagocytes are not harmful but constitute a first line of immunity (innate defense) by virtue of their capacity to ingest and digest invading organisms. By 1908, when the Nobel prize was awarded jointly to Metchnikov and Ehrlich, the late founders of phagocytic (innate) and humoral (adaptive) immunity, productive research on innate immunity had already ebbed, while research on adaptive immunity began to flourish.

One would presume Metchnikov to smile upon realizing the exciting prospects ahead in deciphering the rules governing innate immunity. The battle of innate versus adaptive immunity was and is in vain, since innate immunity indeed is first and effectively controls its child adaptive immunity, endowed with memory and born for the tantalizing task of achieving long-lasting sterilizing immunity to infectious diseases.

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Note Added in Proof

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Immunology

Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization

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ABSTRACT **Recent advances in our understanding of the immune response are allowing for the logical design of new approaches to cancer immunization. One area of interest is the development of new immune adjuvants. Immunostimulatory oligodeoxynucleotides containing the CpG motif (CpG ODN) can induce production of a wide variety of cytokines and activate B cells, monocytes, dendritic cells, and NK cells. Using the 38C13 B cell lymphoma model, we assessed whether CpG ODN can function as immune adjuvants in tumor antigen immunization. The idiotype served as the tumor antigen. Select CpG ODN were as effective as complete Freund's adjuvant at inducing an antigen-specific antibody response but were associated with less toxicity. These CpG ODN induced a higher titer of antigen-specific IgG2a than did complete Freund's adjuvant, suggesting an enhanced TH1 response. Mice immunized with CpG ODN as an adjuvant were protected from tumor challenge to a degree similar to that seen in mice immunized with complete Freund's adjuvant. We conclude that CpG ODN are effective as immune adjuvants and are attractive as part of a tumor immunization strategy.**

Bacterial DNA is capable of inducing activation of B cells, NK cells, and monocytes (1–5). In addition, bacterial DNA can induce production *in vitro* and *in vivo* of a variety of proinflammatory cytokines (6–8). In contrast, vertebrate DNA does not induce lymphocyte activation. Bacterial DNA contains a much higher frequency of unmethylated CpG dinucleotides than does vertebrate DNA due to (*i*) CpG suppression (the under representation of CpG in vertebrate genomes) and (*ii*) methylation of 80% of the CpG in vertebrates. It is possible that lymphocyte activation by the CpG motif in bacterial DNA represents an immune defense mechanism that can distinguish bacterial from host DNA (1). Select synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) have immunologic effects similar to those seen with bacterial DNA. CpG ODN can stimulate monocytes, macrophages, and dendritic cells that then produce several cytokines, including the TH1 cytokine interleukin 12. This effect synergizes with CpG ODN to induce NK cell production of interferon γ (6). Both human and murine leukocytes respond to this novel pathway of immune activation, although individual CpG ODN differ somewhat in their ability to activate various immune cell populations and induce cytokine production in human and murine systems.

The molecular mechanisms responsible for CpG ODN-induced immune cell activation are still under investigation. We have recently reported that CpG ODN trigger the production of reactive oxygen species that activate NF-κB (9). This activation, in turn, leads to cellular activation. Irrespective of the mechanism involved, it is clear that select CpG ODN can have powerful immunologic effects and might be useful therapeutic agents in a number of circumstances, including cancer immunotherapy. For example, we have demonstrated that CpG ODN can enhance antibody-dependent cellular cytotoxicity and improve the *in vivo* efficacy of monoclonal antibody therapy in a syngeneic murine lymphoma model (10).

CpG ODN can induce activation of antigen-presenting cells and enhance production of cytokines known to participate in the development of an active immune response. They also enhance B cell activation, particularly when the B cell receptor is cross-linked (1). These effects are likely to promote antigen-specific responses. Indeed, Branda *et al.* (11) demonstrated that an anti-sense ODN, which in retrospect is noted to contain the CpG motif, enhances antibody response to antigen. We therefore used a well-established animal model to assess whether CpG ODN can function as an immune adjuvant in antitumor immunization.

METHODS

Tumor Model. The 38C13 murine B cell lymphoma model has been used extensively in studies of antibody-based therapy and active immunization (12–15). The idiotype (Id) of the 38C13 surface IgM serves as a highly specific tumor-associated antigen (17, 18). 38C13 Id was obtained from the supernatant of a cell line that secretes 38C13 IgM as described (18), and purified by protein A affinity chromatography. Purified Id was conjugated to keyhole limpet hemocyanin (KLH) using gluteraldehyde and used as the immunogen.

Immunization. Female C3H/HeN mice, obtained from Harlan-Sprague-Dawley, were housed in the University of Iowa Animal Care Unit and used at 6–9 weeks of age. Mice were immunized with 50 μg Id-KLH in a total volume of 200 μl PBS with the indicated antigen and injected subcutaneously except where indicated. Phosphorothioate ODN were produced in a GMP facility and purchased commercially (Oligos Etc., Wilsonville, OR). All CpG ODN were tested for endotoxin content (BioWhittaker), which was undetectable. All CpG ODN cytosines were unmethylated unless indicated. When CpG ODN was used as an adjuvant, both the antigen and CpG ODN were in aqueous phase. When CFA (Sigma) was used as an adjuvant, antigen and complete Freund's adjuvant (CFA) were homogenized before injection.

ELISA Determination of Anti-Id Levels. Serum was obtained by retroorbital puncture from mice following inhalation

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Abbreviations: CpG ODN, oligodeoxynucleotides containing the CpG motif ODN; Id, idiotype; KLH, keyhole limpet hemocyanin; CFA, complete Freund's adjuvant.

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anesthesia with metophane. Microtiter plates were coated with 5 μg/ml 38C13 IgM or irrelevant IgM overnight. IgM-coated plates were blocked with 5% milk, and serial dilutions of serum were added. Plates were washed, and heavy chain-specific goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates) added following by the colorimetric substrate *p*-nitrophenylphosphate. Serum from naive mice to which a known concentration of monoclonal anti-Id was added served as a standard. Plates were evaluated using a microplate reader and curves established for each sample. Test curves were compared with standard curves to determine the concentration of anti-Id. Values were considered valid only if the standard curves and the sample curves had the same shape.

***In Vivo* Survival Studies.** Two weeks after a single subcutaneous immunization, mice were inoculated i.p. with 1,000 38C13 cells. Mice that developed tumor displayed inguinal and abdominal masses, ascites, and cachexia. All mice that developed tumor died. Survival was determined, and significance with respect to time to death was assessed using Cox regression analysis.

RESULTS

CpG ODN 1758 Was Most Effective as an Adjuvant at Enhancing Production of Anti-Id Following Immunization with Id-KLH. Three CpG ODN, *Escherichia coli* DNA, and calf thymus DNA were evaluated for their *in vivo* adjuvant effect. The CpG ODN were selected as representative based on our prior studies of their effects on B cells, cytokine secretion, and induction of NK activity. The sequences for these CpG ODN and their previously described *in vitro* effects are listed in Table 1. C3H mice were immunized with a single subcutaneous injection of 50 μg of Id-KLH in PBS mixed in aqueous solution with 50 μg of CpG ODN or DNA. Serum was obtained weekly and evaluated by ELISA for the presence of antigen-specific antibody (anti-Id IgG) and nonspecific anti-IgM (i.e., rheumatoid factor). As illustrated in Fig. 1, CpG ODN 1643 had a modest effect on enhancing anti-Id IgG levels. CpG ODN 1758 was most effective as an immune adjuvant at inducing production of anti-Id IgG. CpG ODN 1812, which is identical to 1758 in sequence but contains methylcytosines instead of unmethylated cytosines as part of the CpG dinucleotides, had little effect. No nonspecific anti-IgM was noted in any samples (data not shown), demonstrating the antibody response was directed against the idiotype of the 38C13 IgM. The development of an anti-Id response was somewhat higher after subcutaneous and intradermal injection of the antigen and adjuvant when compared with intraperitoneal administration (Fig. 2). There also was a dose-response effect with a maximum effect reached at a dose of 50 μg/mouse (Fig. 3).

The Adjuvant Effect of CpG ODN Is Local, Not Systemic. We next assessed whether the adjuvant effect of CpG ODN was local or systemic. One group of mice were immunized subcutaneously with Id-KLH on one flank and simultaneously injected with 50 μg CpG ODN 1758 on the contralateral flank, whereas a second group received a single injection of both agents (Fig. 4). Injection of CpG ODN and antigen on the same flank was required for maximal adjuvant effect. Thus, CpG ODN exerts much of its adjuvant effect locally. This finding is consistent with our prior observations that footpad injection with CpG ODN enhances NK activity of cells in the ipsilateral but not contralateral lymph node (19).

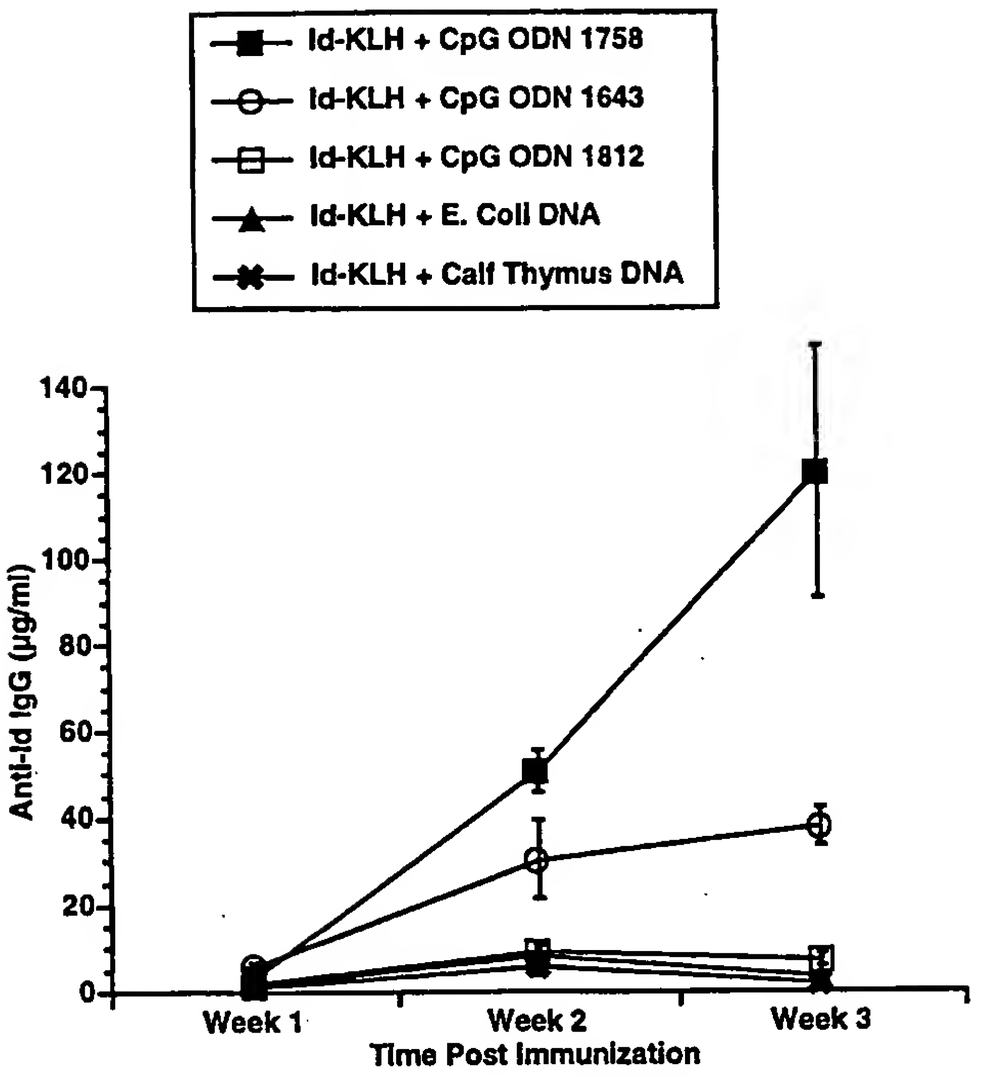


FIG. 1. Comparison of anti-Id titers following immunization using CpG ODN or DNA as an adjuvant. 38C13 Id IgM was conjugated to KLH using gluteraldehyde to produce Id-KLH. A total of 50 μg Id-KLH was mixed with adjuvant in aqueous solution and injected subcutaneously into naive 6- to 8-week-old female C3H mice in a volume of 0.2 ml. Each mouse received a single immunization. Blood was obtained weekly, and serum was evaluated for the presence of anti-Id IgG by ELISA. Normal mouse serum supplemented with a known concentration of monoclonal anti-Id was used as a standard. Three mice were included in each group.

CpG ODN Is as Effective as CFA After Primary and Secondary Immunization. We next compared CpG ODN 1758 as an adjuvant to CFA. Mice were immunized with Id-KLH and CpG ODN 1758 or Id-KLH and CFA on day 0 and boosted with Id-KLH and CpG ODN or Id-KLH and incomplete Freund's adjuvant on day 14. Serum was obtained weekly and anti-Id IgG levels determined. Mice immunized with CFA developed inflammatory masses at the sites of immunization, were less mobile, and demonstrated ruffled fur, whereas no such masses or other changes were noted in mice immunized with CpG ODN. No other toxicity was observed in either group. As illustrated in Fig. 5, CpG ODN 1758 and CFA were similar in their ability to induce anti-Id.

CpG ODN Is More Effective Than CFA at Inducing Production of Antigen-Specific Antibody of the IgG2a Isotype. Murine IgG2a is more effective than murine IgG1 at mediating antibody-dependent cellular cytotoxicity, and monoclonal IgG2a has been shown to be more effective as an antitumor agent in animal models of antibody therapy of cancer when compared with an antibody with the identical variable region

Table 1. Different CpG motifs

ODN	Sequence	Predominant *in vitro* effects
1758	TCTCCCAGCGTGCGCCAT	Monocyte activation; enhanced NK activity (10)
1812	TCTCCCAGZGTGZGCCAT	Minimal immunostimulatory effect (10)
1643	GAGAACGCTCGACCTTCGAT	B cell mitogen (9)

CpG dinucleotides are underlined; Z indicates 5-methylcytosine in the control ODN with methylated CpGs, 1812. All oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

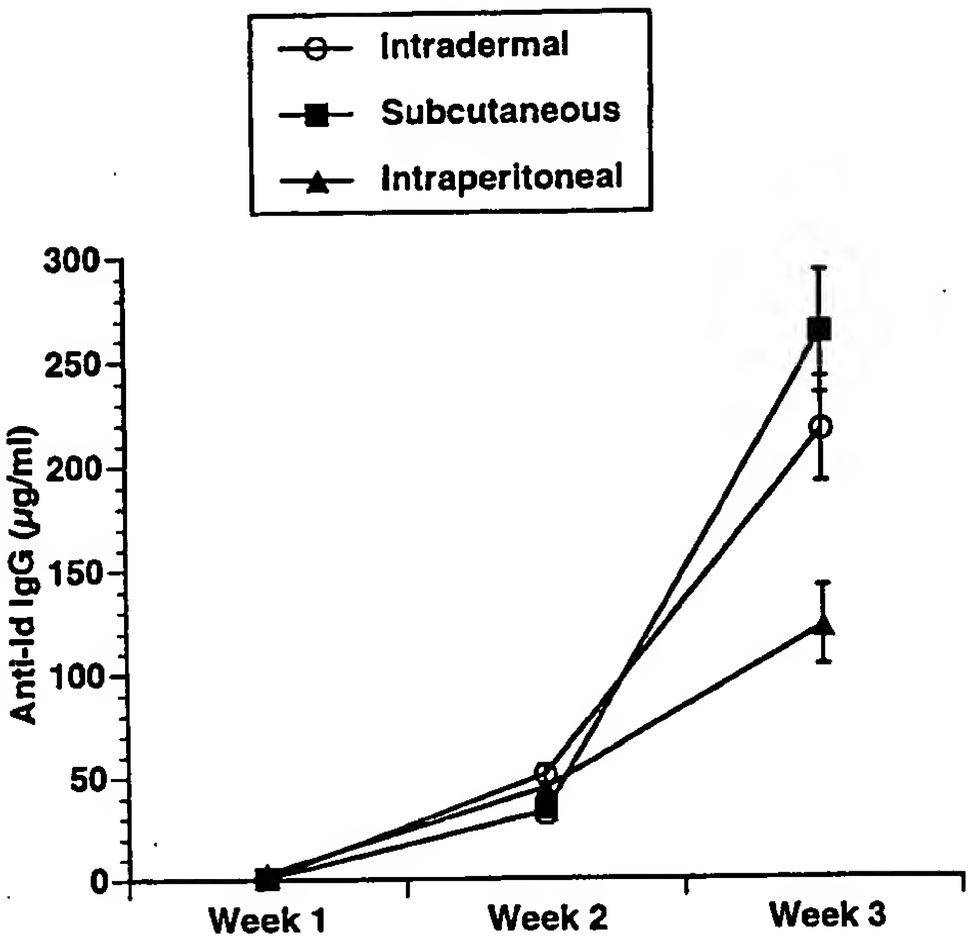
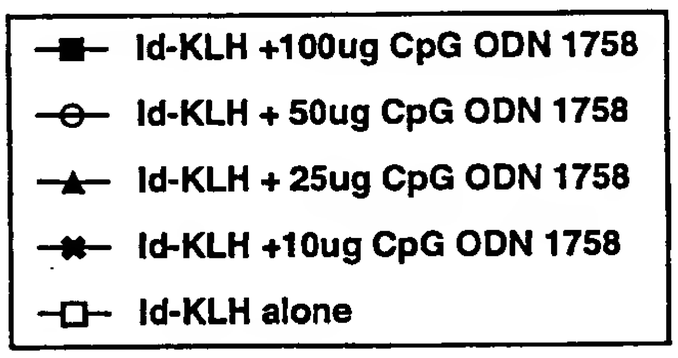


FIG. 2. Route of immunization. Mice were immunized and anti-Id levels determined as in Fig. 1. Development of anti-Id was determined after subcutaneous, intradermal, or intraperitoneal immunization.

of the IgG1 isotype (20). Enhanced production of IgG2a also suggests a TH1 response (21). We therefore evaluated the level of IgG2a and IgG1 anti-Id induced by immunization with CpG ODN 1758, and compared it to that induced by CFA. As shown in Table 2, CpG ODN 1758 induced enhanced production of IgG2a compared with that seen with CFA.

Immunization Using CpG ODN as an Adjuvant Leads to Protection from Tumor Growth. The goal of immunization with tumor antigen is to inhibit tumor growth. We therefore



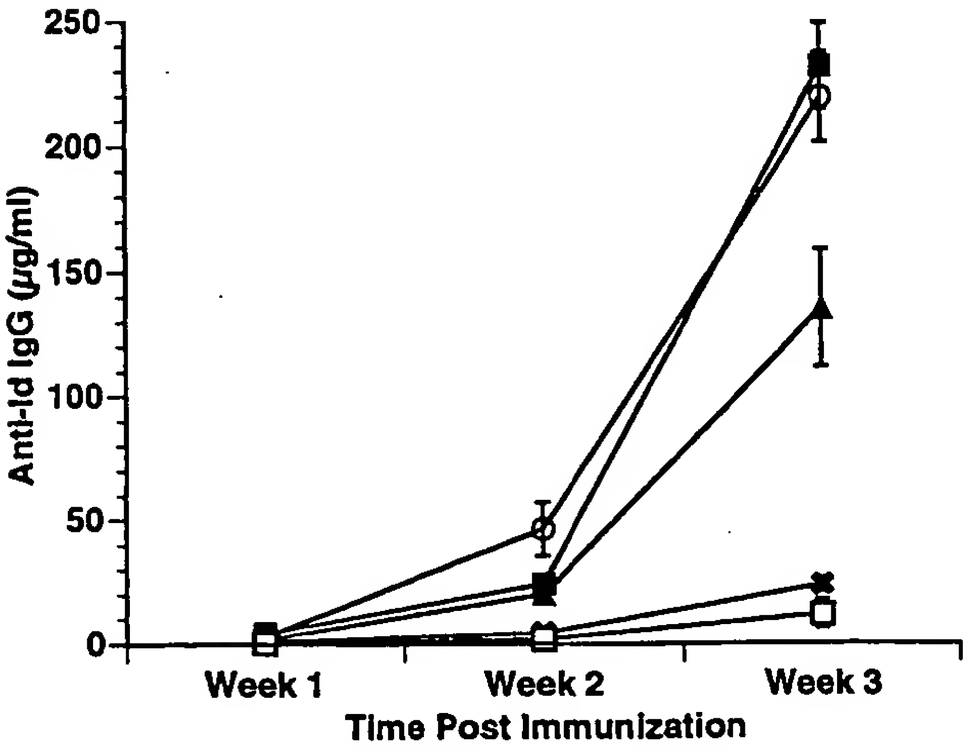


FIG. 3. Dose response to CpG ODN. Mice were immunized and anti-Id levels determined as in Fig. 1. All mice received 50 μg Id-KLH. The dose of CpG ODN 1758 was varied to assess dose response to adjuvant.

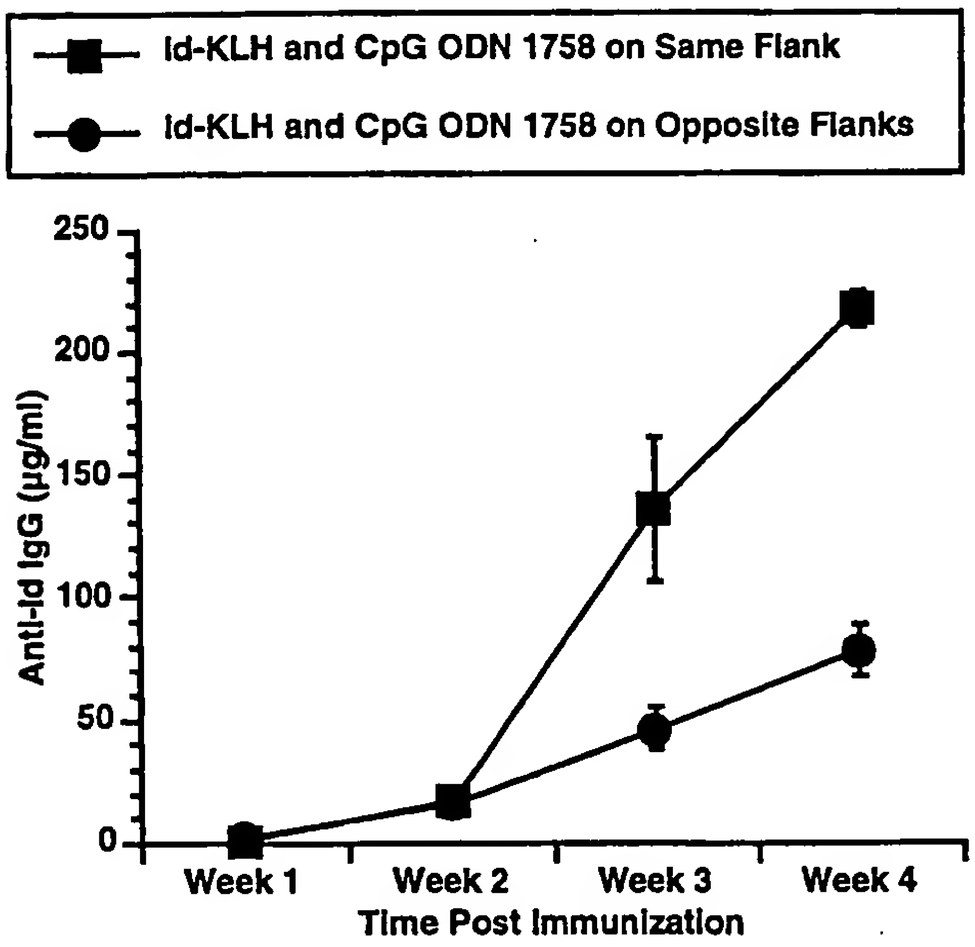


FIG. 4. Comparison of ipsilateral and contralateral injection. Mice were immunized and anti-Id levels determined as in Fig. 1. All mice received 50 μg Id-KLH and 50 μg CpG ODN 1758. Mice were injected with Id-KLH and CpG ODN 1758 mixed together (ipsilateral) or were injected on the right flank with Id-KLH and CpG ODN 1758 on the left flank (contralateral) to assess whether the adjuvant effect of CpG ODN is systemic or local.

evaluated whether 38C13 tumor growth is inhibited in immunocompetent, syngeneic mice immunized with Id-KLH using CpG ODN 1758 as an immune adjuvant. Prior studies in the 38C13 model have involved multiple boosts with Id-KLH in CFA prior to tumor challenge (16). Because we sought to assess whether CpG ODN might be a more effective adjuvant that CFA, we challenged mice with tumor 2 weeks after a single immunization with Id-KLH using CpG ODN 1758 or complete Freund's adjuvant. As illustrated in Fig. 6, all unimmunized mice developed tumor and died within 35 days, with a median survival of 25 days. Mice immunized with Id-KLH and CFA had a median survival of 42 days, and two animals remained disease free indefinitely whereas mice immunized with Id-KLH and CpG ODN 1758 had a median survival of 51 days with four mice remaining disease free. Use of CpG ODN 1758 or CFA as an adjuvant markedly improved survival when compared with control animals ($P < 0.001$). Although mice immunized with CpG ODN 1758 had longer survival than mice immunized with CFA, this difference did not reach statistical significance ($P = 0.18$). CpG ODN 1758 itself without the use of antigen had no detectable protective effect (data not shown).

DISCUSSION

Recent advances in our understanding of tumor immunology and the immune response in general are allowing for the development of new, rational approaches to cancer immunotherapy. One promising approach is immunization with tumor-specific proteins or peptides. Recent studies suggest development of an immune response to a tumor specific antigen after immunization correlates with improved clinical outcome (22). CFA is the standard adjuvant in animal models, although the intensity of the local inflammation that results following injection with this adjuvant prevents its use clinically. Other adjuvants, such as QS21 (23), are currently being evaluated in clinical studies. Recombinant cytokines have also been explored as adjuvants with some success. However, immuniza-

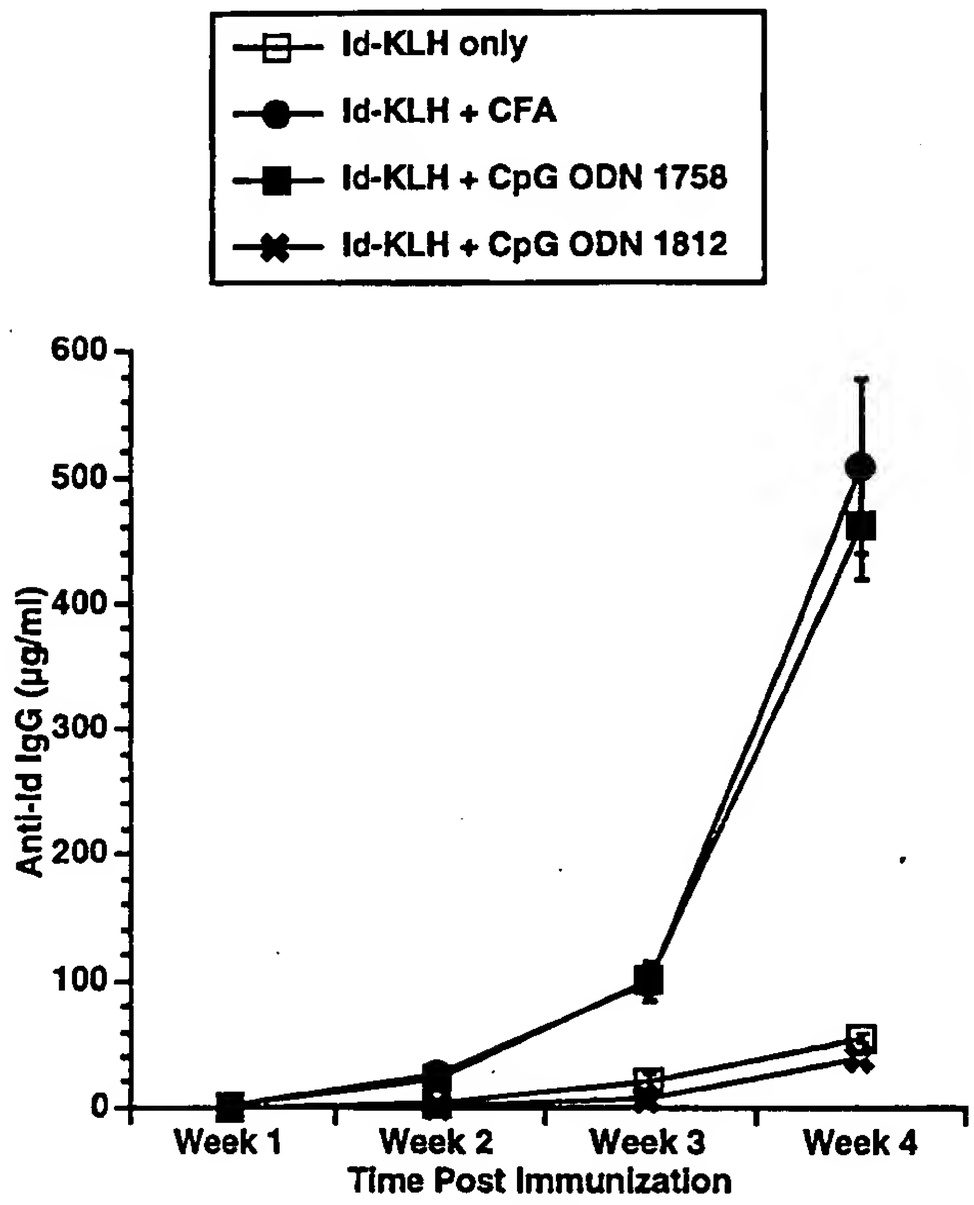


FIG. 5. Comparison of anti-Id titers following immunization with CpG ODN and CFA. Id-KLH was mixed with either CpG ODN 1758 in PBS or homogenized with CFA and injected subcutaneously in a volume of 0.2 ml. Mice were boosted with a second injection at 2 weeks with Id-KLH and CpG ODN 1758 or Id-KLH and incomplete Freund's adjuvant.

tion using an adjuvant that induces the orchestrated activation of various immune subsets and the production of multiple cytokines known to participate in the development of an active immune response is likely to be more effective and perhaps less toxic than immunization using single cytokine as an adjuvant.

Bacterial DNA has significant immunostimulatory effects on B cells, monocytes, and NK cells and can induce production of many of the cytokines that have been shown to be important in the development of antitumor immunity. The studies outlined above confirm that synthetic CpG ODN containing unmethylated CpG motifs can have similar effects. Although our approach is based on the hypothesis that the immune activation in response to bacterial DNA can be used to enhance the immune response to tumor, bacterial DNA itself had no detectable adjuvant effect in our studies. Because of its sensitivity to nucleases, bacterial DNA may not have survived long enough for an adjuvant effect to develop. Indeed, phosphodiester ODN have very short half-lives (24) and would not be expected to have much of an adjuvant effect unless administered repeatedly. The phosphorothioate backbone of the CpG ODN prolongs the half-life of ODN, and this may have

Table 2. CpG ODN 1758 enhances production of antigen-specific IgG2a

Anti-Id levels	CFA	CpG ODN 1758	CpG ODN 1812
IgG2a, µg/ml	54 ± 9	121 ± 17	5 ± 1
IgG1, µg/ml	178 ± 20	127 ± 4	21 ± 2
IgG2a/IgG1	0.30	0.95	0.23

Mice were immunized and serum obtained 3 weeks after a single immunization. Antigen-specific IgG1 and IgG2a was determined by ELISA as described. Data are presented as isotype specific anti-Id ± SEM.

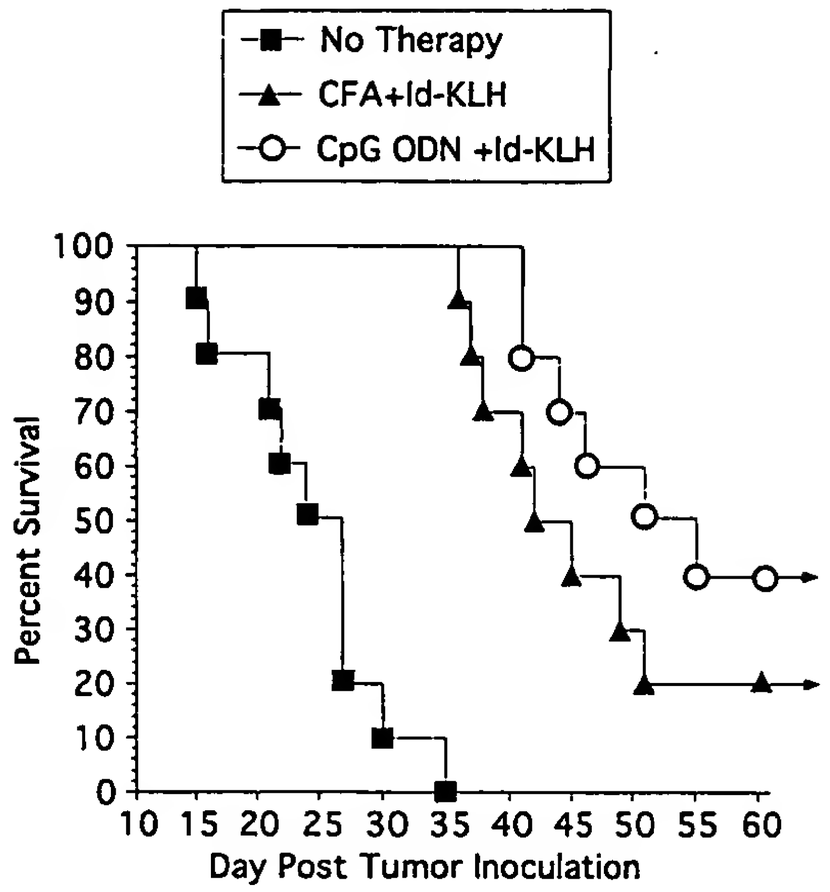


FIG. 6. Tumor protection in C3H mice. Mice were immunized subcutaneously with a single dose of Id-KLH and CpG ODN 1758, Id-KLH, and CFA or were unimmunized. Two weeks later they were challenged with 1,000 38C13 cells intraperitoneally. Survival was followed for 100 days. All mice that were alive after 55 days remained tumor-free for the entire observation period. Ten mice were included in each group.

contributed to its increased efficacy. It is also possible that bacterial DNA activates different subsets of cells than does CpG ODN, and that some negative feedback due to enhanced production of select cytokines, such as TH2 cytokines, limits the adjuvant effect of bacterial DNA. This contention is supported by the fact that CpG ODN 1643 is a more potent B cell mitogen than is CpG ODN 1758, yet had less of an effect as an immune adjuvant.

CpG ODN was as effective as CFA at enhancing production of anti-Id antibody following immunization, and was more effective at inducing production of IgG2a anti-Id. This effect was not seen with an ODN consisting of an identical sequence containing methylated CpG motifs, nor was it as extensive when the CpG ODN was administered on the opposite flank, indicating the adjuvant effect of CpG ODN is secondary to the CpG motif and is largely a local, not a systemic, effect.

A number of important questions remain to be answered. The molecular mechanisms responsible for CpG ODN-induced immunostimulation, and an explanation for why different CpG ODN have different effects, remain unclear and need to be elucidated. The enhanced production of IgG2a suggests a TH1 response to CpG ODN; however, changes in T cell function in response to CpG ODN or enhanced induction of cytotoxic T cells has yet to be explored rigorously. It will be important to determine whether immunization using CpG ODN as an adjuvant indeed enhances the cellular immune response and whether this plays a role in the observed tumor rejection. Synergy with other adjuvants also needs to be explored. Nevertheless, these studies demonstrate that adjuvant CpG ODN can orchestrate an immune response that leads to enhanced antigen-specific antibody formation. CpG ODN could therefore supply a unique approach to enhancing the efficacy of immunization including enhancing antitumor immunity.

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C17

CpG Oligodeoxynucleotides Act as Adjuvants that Switch on T Helper 1 (Th1) Immunity

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Summary

Synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs (CpG ODN) induce macrophages to secrete IL-12, which induces interferon (IFN)-γ secretion by natural killer (NK) cells. Since these cytokines can induce T helper 1 (Th1) differentiation, we examined the effects of coadministered CpG ODN on the differentiation of Th responses to hen egg lysozyme (HEL). In both BALB/c (Th2-biased) and B10.D2 (Th1-biased) mice, immunization with HEL in incomplete Freund's adjuvant (IFA) resulted in Th2-dominated immune responses characterized by HEL-specific secretion of IL-5 but not IFN-γ. In contrast, immunization with IFA-HEL plus CpG ODN switched the immune response to a Th1-dominated cytokine pattern, with high levels of HEL-specific IFN-γ secretion and decreased HEL-specific IL-5 production. IFA-HEL plus CpG ODN also induced anti-HEL IgG2a (a Th1-associated isotype), which was not induced by IFA-HEL alone. Control non–CpG ODN did not induce IFN-γ or IgG2a, excepting lesser increases in B10.D2 (Th1-biased) mice. Thus, CpG ODN provide a signal to switch on Th1-dominated responses to coadministered antigen and are potential adjuvants for human vaccines to elicit protective Th1 immunity.

Antigen-specific CD4+ Th cell responses can be divided into two types, type 1 and type 2, based upon cytokine secretion and effector function (1–3). Type 1 responses involve Th1 cells, whose differentiation is driven by IL-12 (from macrophages) and IFN-γ (from NK cells or T cells). Th1 cells secrete cytokines such as IFN-γ, IL-2, and lymphotoxin. In turn, IFN-γ activates macrophages and enhances immunoglobulin isotype switching to IgG2a, a hallmark of Th1 immunity (4). In contrast, type 2 responses involve IL-4–dependent differentiation of Th2 cells, which produce IL-4, IL-5, IL-10, and IL-13. Type 2 responses are associated with decreased macrophage activation, since some Th2-associated cytokines depress certain macrophage functions. The Th1/Th2 model provides a useful conceptual framework for Th differentiation, and the existence of distinct type 1 and type 2 responses is clearly established, although certain aspects of the model require further investigation (5). Moreover, differential induction of type 1 or type 2 responses is required for protective immunity to certain infectious diseases, and induction of the wrong response type can increase susceptibility to infection (see Discussion). Thus, the type of response induced by a vaccine may be crucial to its efficacy.

The type of Th response generated to an administered antigen can be directed by the type of adjuvant used. Injection of antigen in CFA induces a Th1-dominated response to the antigen, while injection of antigen in IFA induces a Th2-dominated response (6). However, because of its undesirable inflammatory side effects, CFA is not suited for use in human vaccines. Since type 1 immunity plays an important role in the protective response to infection with certain microbes, it is now important to characterize other novel adjuvants that safely induce type 1 immunity and that may potentially be incorporated in future human vaccines. The recent discovery that certain DNA preparations affect cytokine expression by cells of the innate immune system suggests the possibility that DNA preparations could be used as adjuvants to influence the differentiation of Th responses.

The ability of DNA to induce expression of cytokines depends on its source and characteristics (7). In vitro, bacterial DNA induces macrophage expression of IL-12 (8) and TNF-α (9), which are not induced by mammalian DNA. Bacterial DNA also indirectly activates NK cells and stimulates their production of IFN-γ (10–12), since NK cell production of IFN-γ is triggered by IL-12 that is generated by macrophages in response to bacterial DNA (8, 13).

To define components of bacterial DNA that have immunomodulatory effects, a panel of synthetic oligodeoxy-

The first two authors contributed equally to this work.

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nucleotides (ODN)[1] was used to identify specific 6-base pair sequences that conferred activity (14). These sequences shared a CpG motif, containing a central unmethylated CpG dinucleotide preferentially flanked by two 5′ purines and two 3′ pyrimidines. CpG dinucleotides are present in bacterial DNA at the expected frequency of 1/16 bases, but they are three- to fourfold less frequent in mammalian DNA, a phenomenon known as CpG suppression (15). Also, the cytosines in CpG dinucleotides in mammalian DNA are highly methylated, whereas those in bacterial DNA are not (15). Elimination of the CpG sequence or methylation of the cytosine abrogates the stimulatory activity of ODN containing CpG motifs (CpG ODN) and bacterial DNA (9, 11, 14).

When added to splenocytes in culture, CpG ODN induce production of the Th1-associated cytokines IFN-γ and IL-12, as well as the Th2-associated cytokine, IL-6, within several hours (16). However, production of other Th2-associated cytokines, such as IL-4, IL-5, and IL-10, is not detected. The rapid production of IFN-γ is mediated by NK cells stimulated by IL-12 secretion from CpG-activated macrophages; this initial phase of IFN-γ production does not require T cells (8, 13). The induction of IFN-γ and IL-12 (which promote Th1 responses), but not IL-4 (which promotes Th2 responses), suggests that administration of CpG ODN in vivo might produce an environment favoring a Th1 immune response. Indeed, some bacterial plasmid DNA vaccines, which contain this CpG motif, cause development of antigen-specific CD4+ splenocytes that secrete IFN-γ, but not IL-4 or IL-5 (17, 18).

The effect of CpG ODN on antigen-specific T cell responses has not been previously tested. Our current experiments directly test the hypothesis that CpG ODN may serve as adjuvants to switch on Th1 responses. While immunization with hen egg lysozyme (HEL) in IFA induced a Th2-dominated response to HEL, immunization with IFA-HEL plus CpG ODN induced a strongly Th1-dominated response to HEL, as measured by production of specific IgG2a antibody and production of IFN-γ by antigen-stimulated T cells. We propose that CpG ODN function as adjuvants that switch on Th1 responses, making them important candidate adjuvants for potential use in future human vaccines.

Materials and Methods

Oligodeoxynucleotides. ODN were purchased from Operon Technologies (Alameda, CA) or Oligos Etc. (Wilsonville, OR). ODN were phosphorothioate-modified to increase their resistance to nuclease degradation. ODN used in these studies are listed in Table 1 and their sequences are given here (CpG motifs or reversed non-CpG motifs are underlined). Sequences of ODN that were phosphorothioate-modified throughout (S ODN) are: CpG ODN 1826, TCCATGACGTTCCTGACGTT; non–CpG ODN 1745, TCCAATGAGCTTCCTGAGTCT; CpG ODN 1760, ATAATCGACGTTCAAGCAAG; non–CpG ODN 1908, ATAATAGAGCTTCAAGCAAG. Sequences of ODN phosphorothioate-modified on the ends only (S-O ODN) are: CpG ODN 1585, GGGGTCAACGTTGAGGGGGG; and non–CpG ODN 1972, GGGGTCTGTGCTTTTGGGGGG. The first two 5′ end bonds and last five 3′ end bonds of the S-O ODN are phosphorothioate-modified. Synthetic ODN were dissolved in TE (10 mM Tris, 1 mM EDTA). LPS content of ODN was <1 ng LPS/mg DNA, as measured by Limulus amebocyte assay (QCL-1000; BioWhittaker, Walkersville, MD).

Immunizations. BALB/c and B10.D2 mice (Jackson Laboratory, Bar Harbor, ME and Harlan Sprague Dawley, Indianapolis, IN) were housed in microisolators under specific pathogen-free conditions and injected at 7–12 wk of age. HEL (Sigma Chem. Co., St. Louis, MO) was dissolved in PBS, ODN were dissolved in TE, and LPS (*E. coli* 0127:B8; Difco, Detroit, MI) was dissolved in PBS. ODN were added to HEL in a volume less than 10% of the final volume. HEL solutions with or without ODN or LPS were combined with IFA (GIBCO BRL, Gaithersburg, MD) at a 1:1 (vol/vol) ratio and emulsified to achieve a final HEL concentration of 1 mg/ml. CFA was prepared by suspending *Mycobacterium tuberculosis* H37 RA (Difco) at 4 mg/ml in IFA, and CFA was emulsified with the HEL solutions as above. Groups of three mice were injected i.p. with 200 μl of an emulsion and killed 3 wk after injection.

ELISA Assay for Antigen-specific Antibody Production. Sera were collected from mice by tail bleed 3–4 d before sacrifice (15–18 d after immunization with HEL), then diluted 1:10 in PBS/0.2% sodium azide and stored at −20°C. For ELISA, Nunc brand 96-well immunoplates (Fisher, Pittsburgh, PA) were coated by overnight incubation at 4°C with HEL at 10 μg/ml in 0.1 M sodium bicarbonate buffer. Plates were washed and blocked with PBS with 0.05% Tween (PBST) containing 0.1% gelatin for 1–2 h at room temperature. Sera were added to the top row of each plate and serial 1:3 dilutions in PBS were then made into subsequent rows. The plates were incubated overnight at 4°C and washed. Alkaline phosphatase-conjugated detecting antibody was added in PBST/0.1% gelatin and incubated for 2 h at room temperature. For IgG1 and IgG2a detection, goat anti–mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was used at 1:4,000. For total Ig detection, goat anti–mouse Ig(H+L) (Southern Biotechnology Associates), specific for IgM + IgG + IgA, was used at 1:2,000. The colorimetric assay was developed with para-nitrophenyl phosphate (50 mg/ml in 2.5 M sodium bicarbonate/2.5 M magnesium chloride buffer) for 1–3 h. Absorbance at 405 nm was determined using a Beckman Bio Tek Microplate Autoreader (EL309; Beckman Instruments, Palo Alto, CA). The serum from each mouse was assayed in duplicate and the mean value was used to represent each animal. These values were used to calculate the mean and standard deviation for each group of three mice.

ELISA Spot Assay for Cytokine Production. Splenocytes were isolated from mice 3 wk after immunization. A modified ELISA spot assay for detection of cytokine production by splenocytes has been developed in prior work (6). ELISA spot plates (Polyfiltronics, Rockland, MA) were coated with capture antibody for IFN-γ (R46A2; 4 μg/ml in PBS) or IL-5 (TRFK5; 5 μg/ml in PBS) overnight at 4°C. Plates were then washed and blocked with PBS/1% BSA for 1–2 h at room temperature. After washing, freshly isolated splenocytes were plated at 10^6 cells/well in serum-free medium, HL-1 (BioWhittaker), supplemented with

[1] *Abbreviations used in this paper:* HEL, hen egg lysozyme; ODN, oligodeoxynucleotides; PBST, PBS with Tween; S ODN, phosphorothioate-modified ODN; S-O ODN, ODN with partial phosphorothioate modification.

Table 1. *Sequences of Synthetic ODN*

ODN	Sequence*	Motif	Backbone
	↓↓ ↓↓		
1826	TCCATGACGTTCCTGACGTT	CpG	S ODN
1745	TCCAATGAGCTTCCTGAGTCT	non-CpG	S ODN
	↓↓ ↓↓		
1760	ATAATCGACGTTCAAGCAAG	CpG	S ODN
1908	ATAATAGAGCTTCAAGCAAG	non-CpG	S ODN
	↓↓		
1585	GGGGTCAACGTTGAGGGGGG	CpG	S-O ODN
1972	GGGGTCTGTGCTTTTGGGGGG	non-CpG	S-O ODN

Nucleotides that have their 3′ linkage phosphorothioate modified to increase resistance to nuclease degradation are in bold print.
*The CpG motifs or corresponding non-CpG motifs are underlined.

L-glutamine and penicillin/streptomycin, in the presence or absence of HEL (100 μg/ml). In some experiments purified GK1.5 anti-CD4 antibody (American Type Culture Collection, Rockville, MD) was added at 10–30 μg/ml to block CD4 T cell function. After culture for 24 h (for IFN-γ detection) or 48 h (for IL-5 detection), cells were removed by washing with PBS and then PBST. Detecting antibody (XMG1.2-HRP, 1:400 for IFN-γ; TRFK4, 4 μg/ml for IL-5) was added in PBST/1% BSA and incubated overnight. For the IL-5 assay only, anti-IgG2a-HRP (Zymed, South San Francisco, CA) was added after washing with PBST, and the plates were incubated for 2 h at room temperature. All plates were washed with PBS before developing the colorimetric assay by the addition of 1% 3–amino-9-ethylcarbazole/*N,N*-dimethylformamide in 0.1 M sodium acetate buffer (1:30 vol/vol) for 45–60 min. The plates were then washed with distilled water and air dried. Spots were quantitated by an image analysis program (Optimas, Bothell, WA).

Results

Coadminstered CpG ODN Induce Production of HEL-specific IgG2a (a Th1-associated Isotype). BALB/c mice were injected i.p. with 200 μg of HEL in the following adjuvants: CFA, IFA, IFA with CpG ODN 1826, or IFA with a similar ODN lacking the CpG motif, ODN 1745. ODN 1826 and ODN 1745 are phosphorothioate-modified for the entire length of the backbone (S ODN; see Table 1), which greatly increases resistance to nuclease degradation (19). Based on preliminary dose titration studies, ODN were initially used at 100 μg/mouse. Sera were collected 15–18 d after immunization and assayed for anti-HEL Ig (total or specific isotype) by ELISA. Consistent with previous results demonstrating that IFA induces a Th2 response while CFA induces a Th1 response to antigen (6), mice injected with IFA-HEL did not produce detectable IgG2a responses (Fig. 1 *A*). In contrast, mice injected with CFA-HEL produced high levels of IgG2a. The addition of non–CpG ODN 1745 to the IFA-HEL protocol did not induce IgG2a production. However, immunization with IFA-HEL-CpG ODN 1826 altered the isotype profile of the antibody response, causing a marked increase in anti-HEL IgG2a. Furthermore, in three independent experiments, the production of HEL-specific IgG2a was consistently higher in mice treated with IFA-HEL-CpG ODN 1826 than in mice treated with CFA-HEL.

Despite the changes in IgG2a responses, similar levels of anti-HEL IgG1 or total anti-HEL Ig were produced by all immunizations (Fig. 1, *B* and *C*). Thus, immunization with IFA-HEL or IFA-HEL–non-CpG ODN 1745 was successful and sufficient to generate an antibody response to HEL, with both anti-HEL IgG1 and total anti-HEL Ig levels comparable to those seen with CFA-HEL or IFA-HEL-CpG ODN 1826. Although the IgG1 isotype has been linked to Th2 responses, our data demonstrate that IgG1 can also be observed in Th1-dominated responses and, at least in this system, cannot be used to accurately assess Th differentiation. We conclude that the increased IgG2a production associated with IFA-HEL-CpG ODN 1826, like that caused by CFA-HEL, represents a selective induction of this isotype, i.e., a qualitative switch in the relative levels of antibody isotypes produced rather than a simple enhancement of all anti-HEL isotypes.

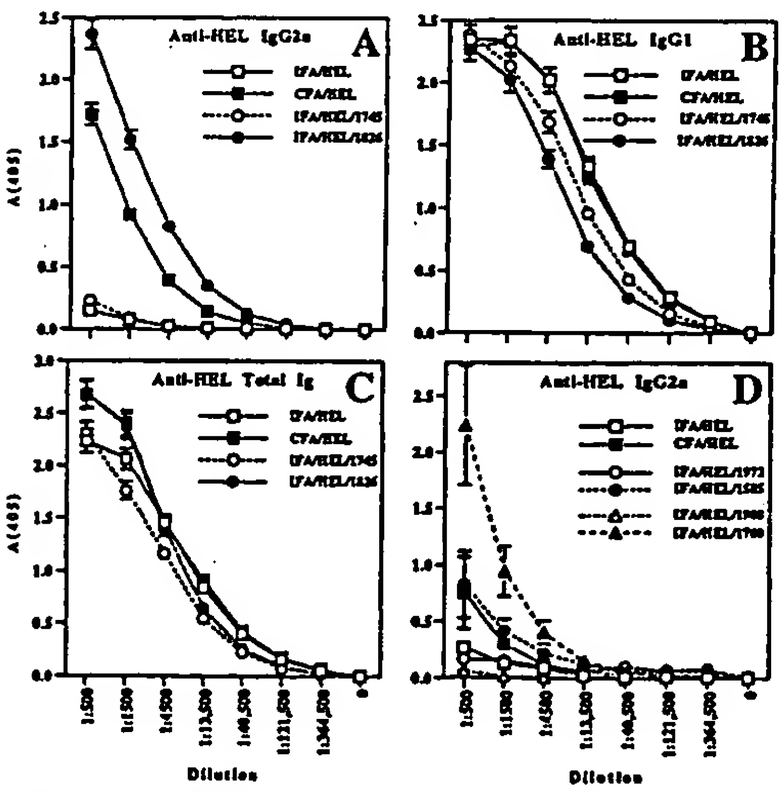

Figure 1. Th1-associated antigen-specific IgG2a responses are induced by immunization of BALB/c mice with IFA-HEL-CpG ODN but not IFA-HEL-non–CpG ODN. (*A–C*). Mice were injected i.p. with CFA-HEL (a control for a Th1-dominated response), IFA-HEL (a control for a Th2-dominated response), or IFA-HEL with 100 μg of CpG ODN 1826 or non–CpG ODN 1745. Sera were collected from mice 15–18 d after injection and assayed by ELISA for: (*A*) anti-HEL IgG2a, an isotype associated with Th1-dominated responses; (*B*) anti-HEL IgG1; and (*C*) anti-HEL total Ig response. *A–C* represent data from a single experiment representative of three similar experiments. (*D*) BALB/c mice were immunized as above, except that 30 μg of CpG ODN 1585, non–CpG ODN 1972, CpG ODN 1760, or non–CpG ODN 1908 was used for each mouse. Anti-HEL IgG2a antibodies were detected by serum ELISA. Data shown in *D* are representative of three similar experiments.

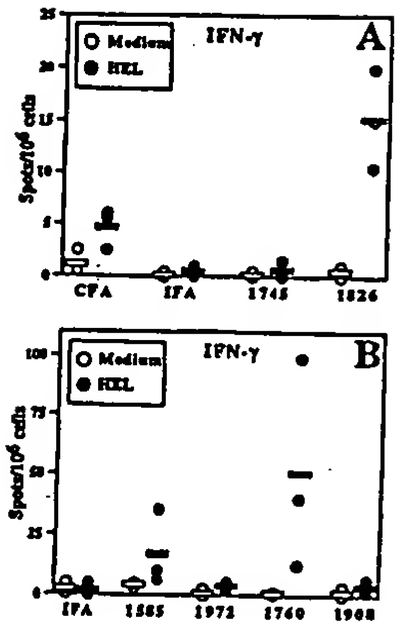

Figure 2. CpG ODN enhance HEL-specific IFN-γ production by BALB/c splenocytes. Mice were immunized as in Fig. 1 with 100 μg ODN/mouse in *A* and 30 μg ODN/mouse in panel B. After 3 wk, splenocytes were isolated and incubated with HEL (*closed circles*) or medium alone (*open circles*). ELISA spot assay was performed and spots were quantitated by a computerized image analysis program. Each point represents the mean number of spots per well for one mouse (assayed in duplicate); horizontal bars indicate the mean of points for each group of mice. Similar results were observed in five independent experiments with CpG- and non-CpG ODN in BALB/c mice.

To confirm the role of the CpG motif, we also examined the effects of two additional pairs of CpG and non-CpG ODN. CpG ODN 1760 and a related non-CpG control, ODN 1908, are S ODN (Table 1). CpG ODN 1585 and a related non-CpG control, ODN 1972, are phosphorothioate-modified on the ends only (S-O ODN; see Table 1). ODN 1760 and ODN 1826 share a common CpG motif, GACGTT. Immunization of BALB/c mice with HEL in IFA with or without 30 μg of each ODN showed that both CpG ODN (ODN 1760 and ODN 1585) induced anti-HEL IgG2a antibodies, which were not induced by the non-CpG ODN (ODN 1908 and ODN 1972) (Fig. 1 *D*). The S ODN 1760 induced significantly higher levels of anti-HEL IgG2a than CFA or the S-O ODN 1585 (which is more nuclease-sensitive than ODN 1760). Anti-HEL IgG1 and total anti-HEL Ig responses were similar in all groups (data not shown), again indicating that all of the immunizations generated anti-HEL antibody responses of similar overall magnitude. We conclude that antigen-specific IgG2a antibodies are induced by CpG ODN, suggesting that CpG ODN induce a Th1-dominated response to coadministered protein antigen.

Coadministration of CpG ODN Induces Th1-dominated Antigen-specific Cytokine Responses. We used a modified ELISA spot assay (see Materials and Methods) to assess recall antigen-specific IFN-γ secretion as a measure of Th1 memory cells induced after immunization with HEL. Three weeks after immunization of BALB/c mice with HEL in adjuvant, splenocytes were isolated, incubated in vitro with or without HEL and assayed for IFN-γ production. Upon restimulation with HEL, splenocytes from mice immunized with IFA-HEL showed little or no antigen-specific production of IFN-γ (Fig. 2 *A*), as expected (6). In contrast, splenocytes from mice immunized with CFA-HEL showed antigen-specific production of IFN-γ, demonstrating that CFA-HEL induced a Th1 response to HEL in these mice, as previously observed (6). Prior studies using isolated spleen-derived CD4+ T cells (with irradiated BALB/c-*scid* splenocytes as antigen-presenting cells) have shown that antigen-specific cytokine secretion measured by this assay is mediated by CD4+ T cells (Yip, H., A. Karulin, M. Tary-Lehmann, P. Heeger, R. Trezza, T. Forsthuber, and P.V. Lehmann, manuscript submitted for publication).

The results obtained with ODN established an important role for the CpG motif in determining Th differentiation. Immunization with IFA-HEL-non-CpG ODN 1745 did not enhance antigen-specific IFN-γ production over that observed with IFA-HEL. In contrast, immunization with IFA-HEL-CpG ODN 1826 strongly induced the production of antigen-specific cells secreting IFN-γ (Figs. 2 *A* and 3). Immunization with IFA-HEL-CpG ODN 1826 produced two- to fourfold more antigen-specific IFN-γ secretion than observed with CFA-HEL (Fig. 2 *A* and data not shown).

To determine the dose range for effective Th1 adjuvant activity of CpG ODN 1826, BALB/c mice were injected i.p. with 200 μg HEL in IFA, together with 0, 10, 30, or 100 μg ODN 1826. Antigen-specific serum Ig levels were assayed as above. Production of anti-HEL IgG2a was strongly enhanced in mice treated with as little as 10 μg ODN 1826, while specific production of total Ig and IgG1 was not affected by ODN 1826 at any dose (data not shown). ELISA spot analysis of splenocytes from these mice showed strong induction of IFN-γ by 30 or 100 μg ODN 1826 and lesser enhancement with only 10 μg (data not shown). Thus, Th1-directing adjuvant activity of CpG ODN is seen with doses as low as 10 μg in BALB/c mice.

To confirm that the Th1 adjuvant activity of CpG ODN 1826 was specific to the CpG motif, other CpG and non-CpG ODN were examined for effects on the differentiation of the Th response to HEL. As demonstrated in Fig. 2 *B*, immunization with both of the additional CpG ODN (ODN 1760 and ODN 1585) increased the number of cells secreting IFN-γ in response to secondary stimulation with HEL, while little or no increase was seen with the non-CpG ODN (ODN 1972 and ODN 1908). Consistent with the pattern of IgG2a induction, the S ODN 1760 appeared to have a greater effect than the S-O ODN 1585 on increasing numbers of IFN-γ–secreting cells. Thus, the number of cells secreting IFN-γ is enhanced by CpG ODN but not by non-CpG ODN, supporting the induction of Th1-dominated responses by CpG ODN. Furthermore, the addition of anti-CD4 antibody (GK1.5 at 10–30 μg/ml) during the in vitro antigen stimulation blocked CpG ODN-enhanced, HEL-specific IFN-γ secretion (data not shown), confirming that the CpG ODN-enhanced production of IFN-γ was T cell–dependent in this system.

To assess Th2 differentiation, ELISA spot analysis was similarly performed to detect splenocytes producing IL-5 (Fig. 4). Immunization with IFA-HEL induced cells that secreted IL-5 in response to restimulation with HEL, consistent with a Th2-dominated response. In contrast to the results with IFA-HEL, little or no HEL-specific IL-5 secretion was seen in mice immunized with CFA-HEL, consistent with a Th1-dominated anti-HEL response in these mice. HEL-specific IL-5 secretion was observed after im-

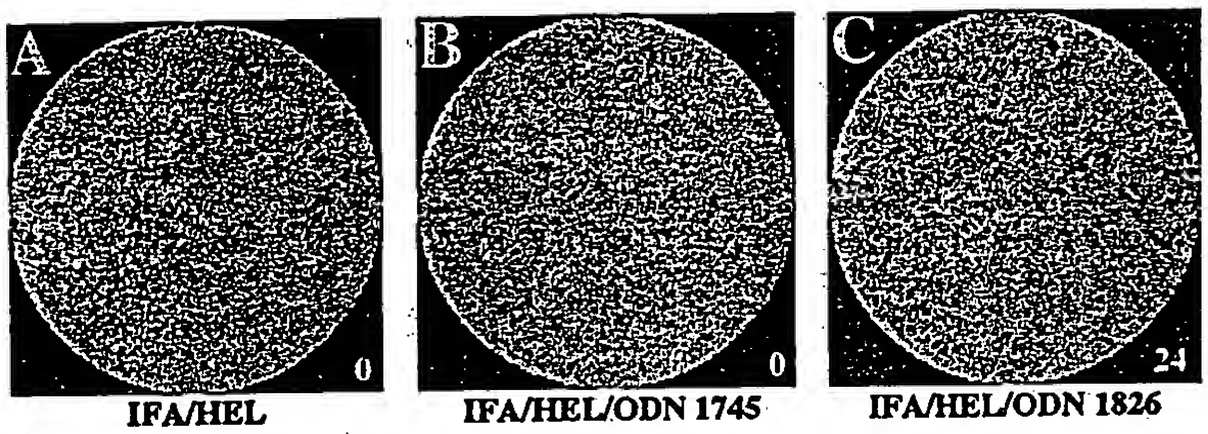

Figure 3. ELISA spot assessment of IFN-γ production by splenocytes from immunized BALB/c mice. Pictures show representative images of ELISA spot wells from the experiment shown in Fig. 2 *A*. The number of spots, as quantitated by an image analysis program, is indicated next to each well. Each well contained HEL (100 μg/ml) and 10^6 splenocytes isolated from mice immunized with IFA-HEL (*A*), IFA-HEL-non-CpG ODN 1745 (*B*) or IFA-HEL-CpG ODN 1826 (*C*).

munization with IFA-HEL-non-CpG ODN (e.g., ODN 1745 and ODN 1972), although immunization with IFA-HEL-non-CpG ODN 1745 produced somewhat lower levels of IL-5 than observed with IFA-HEL. In contrast, greater reduction in HEL-specific IL-5 secretion was observed after immunization with CpG ODN (ODN 1826, ODN 1760 and ODN 1585). Thus, the addition of CpG ODN induced a switch from a Th2-dominated response to a Th1-dominated response, as manifested by a decrease in Th2-associated cytokine secretion as well as the induction of Th1-associated cytokine secretion.

Together, these results indicate that CpG ODN directed Th1 differentiation in the T cell responses to coadminstered antigen. Relative to immunization with IFA-HEL, immunization with IFA-HEL-CpG ODN increased HEL-specific IFN-γ production by splenocytes, decreased HEL-specific IL-5 production by splenocytes and increased IgG2a anti-HEL titers. Furthermore, the Th1 adjuvant activity of CpG ODN for both antigen-specific antibody and cytokine production was significantly greater than that of an established Th1 adjuvant, CFA.

CpG ODN Direct Th1-dominated Responses in Th1-biased (B10.D2) Mice as well as Th2-biased (BALB/c) Mice. Strains of mice differ in genetic bias toward the development of Th1- or Th2-dominated Th responses. Earlier publications have demonstrated that BALB/c mice are Th2-biased, while B10.D2 mice are more Th1-biased (20). To explore the impact of varying Th1/Th2 bias on the effect of CpG ODN, B10.D2 mice were injected i.p. with IFA-HEL, with or without 30 μg CpG ODN 1826 or non-CpG ODN 1745, and splenocytes were subsequently isolated for ELISA spot analysis. Immunization with IFA-HEL-CpG ODN 1826 produced a very high level of HEL-specific IFN-γ production, while IFN-γ was not produced after immunization with IFA-HEL alone (Fig. 5). Again, CpG ODN 1826 induced levels of HEL-specific IFN-γ production that exceeded even those seen after immunization with CFA-HEL, and augmentation of IFN-γ production, albeit at lower levels, was seen in B10.D2 mice treated with as little as 3 μg of ODN 1826 in IFA-HEL (data not shown). Immunization with IFA-HEL plus either of the two other CpG ODN, ODN 1760, and ODN 1585, also induced HEL-specific secretion of IFN-γ (data not shown). Immunization with the non-CpG ODN 1745 and 1908 (30 μg dose) induced HEL-specific production of IFN-γ by splenocytes from B10.D2 mice, but at a minimal level (Fig. 5 and data not shown), while the other non-CpG ODN, ODN 1972, did not induce IFN-γ (data not shown). Thus, CpG ODN had strong Th1 adjuvant activity in Th1-biased as well as Th2-biased mice, while non-CpG ODN induced little or no Th1 differentiation, as assessed by antigen-specific secretion of IFN-γ.

The effects of CpG ODN on IFN-γ responses were paralleled by changes in IgG2a levels in B10.D2 mice. Again, immunization with IFA-HEL-CpG ODN 1826 (3, 10, or 30 μg ODN) induced high titers of anti-HEL IgG2a, and similar results were seen with the other CpG ODN, ODN

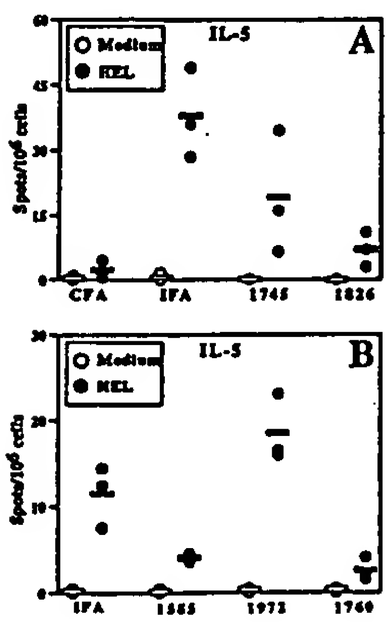

Figure 4. CpG ODN decrease HEL-specific IL-5 production by BALB/c splenocytes. Mice were immunized as in Fig. 2 (30 μg ODN/mouse), and splenocytes were harvested for in vitro restimulation with or without HEL. ELISA spot analysis was performed for IL-5. The data are representative of five similar experiments with CpG- and non-CpG ODN in BALB/c mice.

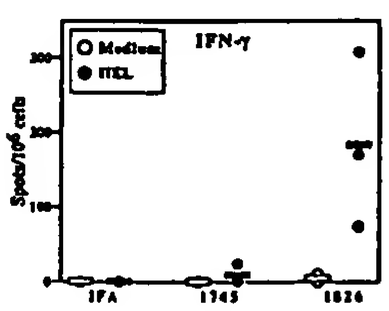

Figure 5. Induction of HEL-specific IFN-γ responses by CpG ODN in B10.D2 mice. B10.D2 mice were immunized as in Fig. 1, except that ODN were used at 30 μg per mouse. Three weeks after immunization, HEL-specific production of IFN-γ by splenocytes was measured by ELISA spot assay as in Fig. 2. The data shown are representative of three similar experiments.

1760, and ODN 1585 (only the 30 μg dose was assessed, data not shown). Mice treated with 30 μg non-CpG ODN 1745 also produced anti-HEL IgG2a, though the levels were not as high as in mice treated with ODN 1826, but the other non-CpG ODN (ODN 1908 and ODN 1972) did not induce IgG2a production. Anti-HEL total Ig and IgG1 levels were similar under all of these conditions (data not shown). These results confirm that CpG ODN enhance Th1-associated antigen-specific IgG2a responses in both Th1- and Th2-biased mouse strains.

Discussion

Because it is highly effective in inducing both cellular and humoral immunity, CFA has been an important model adjuvant (21). Furthermore, CFA has been shown to induce Th1-dominated immune responses (6). However, due to its inflammatory side effects, CFA cannot be used in humans. Thus, the discovery and characterization of adjuvants that promote Th1 cell-mediated immunity is currently an important area in vaccine development. Our results establish that CpG ODN are excellent candidate adjuvants for vaccines to induce Th1 immunity.

CFA is prepared by mixing two components, IFA (mineral oil) and nonviable *Mycobacterium tuberculosis*. As an adjuvant, CFA has been proposed to provide two crucial functions. First, it creates a local antigen depot (by entrapment of antigen in the mineral oil emulsion) which allows for prolonged regional antigenic stimulation. This function is also provided by IFA. Second, CFA contains immunomodulatory substances derived from *Mycobacterium tuberculosis* that stimulate immune responses and promote Th1 differentiation. It is possible that the adjuvant activity of CFA may be due in part to mycobacterial DNA, as *M. bovis* DNA sequences have been shown to be immunostimulatory (22, 23). Thus, a general strategy for the development of type 1 vaccine adjuvants may be to provide both an antigen depot and an immunomodulatory function that promotes Th1 differentiation.

Our studies address the ability of CpG ODN to modulate the differentiation of Th responses. In these experiments, ODN were mixed with IFA, which itself establishes an antigen depot but does not promote Th1 differentiation (future vaccines using CpG ODN may include alternative components such as biodegradable oils to optimize vaccine safety and efficacy). Mice that were immunized with IFA-HEL developed both humoral and Th2 cellular immune responses, consistent with prior observations (6). However, the addition of CpG ODN to this system induced strong Th1-dominated responses. Th1-dominated responses were induced with as little as 3 μg CpG ODN in B10.D2 mice and 10 μg CpG ODN in BALB/c mice.

As a direct measure of Th1 differentiation, we monitored the number of splenocytes secreting IFN-γ after immunization and in vitro stimulation with HEL by ELISA spot assay. This assay has been shown to detect cytokine secretion by individual CD4+ T cells (Yip, H., A. Karulin, M. Tary-Lehmann, P. Heeger, R. Trezza, T. Forsthuber, and P.V. Lehmann, manuscript submitted for publication). Also, addition of a blocking anti-CD4 mAb to the splenocytes during in vitro stimulation with HEL decreased HEL-specific production of IFN-γ by >80% (data not shown), confirming that the production of IFN-γ was dependent on antigen-specific CD4+ T cells. We also measured IFN-γ production by standard ELISA and found the same pattern of results as obtained by the ELISA spot assay (data not shown).

The addition of CpG ODN as adjuvants produced levels of IFN-γ that surpassed even the levels observed with CFA. Two of the non-CpG ODN (ODN 1745 and ODN 1908) induced IFN-γ secretion, but only at minimal levels and only in Th1-biased B10.D2 mice. ODN 1745 also induced production of antigen-specific IgG2a, although ODN 1908 did not. The slight CpG-like effects of ODN 1745 may be due in part to the presence of TG dimers in a context that provides a weak analogue of a CpG motif. Other studies have shown that weak induction in vitro of other CpG-like effects by ODN 1745 (B cell proliferation, secretion of IL-6, TNF-α, and IL-12) are eliminated in analogues of ODN 1745 that lack TG dimers (Krieg, A.M., unpublished observations). In addition, weak CpG-like effects could be triggered by the DNA backbone of non-CpG ODN (e.g. ODN 1745 and ODN 1908), since the phosphorothioate backbone of modified ODN has some intrinsic immunostimulatory properties (7, 24). Despite minor activities of some non-CpG ODN, the CpG ODN were vastly superior for inducing Th1-dominated immune responses in both Th1- and Th2-biased mouse strains. These observations indicate that the CpG motif provides a strong and reliable stimulus for Th1 differentiation in animals of differing genetic background.

We also assessed the differentiation of Th responses indirectly by the isotype profile of antigen-specific antibody responses. CpG ODN induced levels of antigen-specific IgG2a that surpassed even those achieved with CFA. Total anti-HEL Ig and anti-HEL IgG1 levels induced by CpG ODN did not differ appreciably from those induced by IFA or CFA, indicating that all of the immunization protocols used in this study were successful and effective for generation of anti-HEL Ig. Thus, the increased production of IgG2a induced by CpG ODN and CFA represents a qualitative switch in Ig isotype production from an IFA-induced Th2-influenced pattern to a Th1-influenced pattern.

We assessed the differentiation of Th2 responses by antigen-specific IL-5 production. Although IL-4 is also produced during Th2 responses, there can be differences in the production and source of these two cytokines under some circumstances. Even in the context of a Th1 response, antigen-stimulated T cells can induce IL-4 (but not IL-5) secretion by non-T cells (Karulin and Lehmann, unpublished observations). Other groups have reported that treatment with IL-12 at the time of immunization can induce antigen-specific IL-4 production by splenocytes (25), again demonstrating that IL-4 can be detected alongside markers of Th1

responses. Therefore we chose IL-5 secretion as a clear, specific marker for Th2 differentiation in our studies, i.e., a cytokine whose antigen-triggered secretion could be attributed to antigen-specific T cells. The differentiation of cells secreting IL-5 was monitored by the ELISA spot assay. We also tested the supernatants from similar incubations for IL-5 by standard ELISA, which revealed the same pattern of results (data not shown).

ELISA spot analysis demonstrated that HEL-specific IL-5 secretion was induced by immunization with IFA-HEL but was absent after immunization with CFA-HEL. Immunization with IFA-HEL-CpG ODN produced significantly lower levels of IL-5 secretion than observed with IFA-HEL. Thus, in addition to enhancing Th1 differentiation, CpG ODN appear to at least partially switch off Th2 differentiation to produce Th1-dominated immune responses. Moreover, these studies examined Th differentiation after a single immunization. It is possible that repeated administration of antigen with CpG ODN would produce an even more polarized Th1 response.

The experiments shown here monitored IgG2a levels at 15-18 d after immunization and T cell responses at three weeks after immunization. Additional experiments also examined cytokine responses upon antigenic restimulation of T cells at 4 and 6 wk after immunization, as well as IgG2a levels at 3–4 d before these times. At all of these time points the exact same pattern of results was consistently found. For example, CpG ODN produced enhanced IFN-γ and IgG2a responses in all experiments at these later time points (data not shown). These results indicate that the pattern of T cell differentiation induced by a single immunization with CpG ODN remains stable for at least 6 wk.

The possibility that the effects of CpG ODN were due to contaminating LPS was excluded by the following observations. First, LPS levels in the ODN preparations were very low (<<1 ng LPS/mg DNA). Second, we immunized with IFA-HEL plus LPS at 1 ng/mouse (an amount 10–100-fold higher than the maximum amounts contributed by the highest ODN doses) and failed to see any increase in Th1-associated results (IFN-γ secretion and induction of IgG2a) or decrease in Th2-associated IL-5 secretion (data not shown).

One concern for DNA adjuvants, as with all adjuvants, is the potential for toxicity. The administration of bacterial DNA or stimulatory ODN can induce TNF-α release and fatal shock in mice that have been previously sensitized with D-galactosamine (26), and CpG DNA can prime mice for the Shwartzmann reaction (12). With regard to ODN adjuvants, mice given repeated high doses of CpG ODN develop a dose-dependent splenomegaly and can develop other toxicity related to excessive immune stimulation, including death (27). However, significant toxicity has not been observed at the low doses of ODN used for adjuvant function in our current studies, where we observed no significant changes in mouse appearance, behavior, and body weight (measured at multiple points throughout the experiment) or spleen weight (measured at the time of sacrifice). We also noted that spleens from mice injected with CFA were more difficult to remove, presumably due to post-inflammatory peritoneal fibrosis, while spleens from mice injected with CpG ODN appeared normal. Additional studies of spleen and lymph node sizes at earlier time points after administration of CpG ODN alone, at the doses used here, revealed mild splenomegaly and hyperplasia of draining lymph nodes that was reversible within 10–14 d. Mice injected with a single dose of up to 1 mg of the S ODN used in these studies (100-fold higher than the effective adjuvant dose) showed no apparent systemic toxicity or change in feeding, grooming, physical activity or behavior. We conclude that CpG ODN provide potent adjuvant activity at doses that produce no dangerous toxicity.

In the context of vaccine development, the ability to direct Th1 or Th2 differentiation of antigen-specific immune responses has significant implications for therapy of various infectious and autoimmune diseases (28). In the case of certain infectious diseases, Th1-dominated immune responses are protective, while Th2-dominated responses are associated with disease susceptibility. For example, in murine leishmaniasis, Th2-biased mouse strains (e.g., BALB/c) make IL-4–dominated responses to parasite antigens and are susceptible, whereas mice that mount Th1-dominated responses involving IFN-γ secretion (e.g., C57BL/6) are resistant (29–31). Moreover, susceptible mice can be made resistant by administration of IL-12 to enhance Th1 immunity or antibody blockade of IL-4 (32-34), and resistant mice can be made sensitive by blockade of IL-12 (35). In similar circumstances in humans, the ability to direct vaccine-induced immunity towards Th1 responses will dictate the success of vaccination. Thus, CpG ODN may be useful as adjuvants to induce protective Th1 immunity.

In other circumstances the utility of CpG ODN may lie in their potential ability to redirect pathogenic Th2 responses to less harmful Th1 responses. For example, Th2-dominated responses appear to cause allergy, and recent data suggest that administration of CpG ODN may prevent or even reverse ongoing allergic reactions, presumably by redirecting a Th2-dominated response to allergen (which promotes IgE synthesis) to a Th1-dominated response (Kline, J., T. Businga, T. Waldschmidt, J. Weinstock, and A.M. Krieg, manuscript submitted for publication). Similarly, autoimmune diseases that are potentially Th2-associated, such as systemic lupus erythematosis, may be amenable to such Th1 therapy. Th1 therapy, however, is potentially associated with the danger of inducing Th1-mediated pathology, such as certain Th1-associated autoimmune diseases (e.g., experimental allergic encephalomyelitis and type I insulin dependent diabetes mellitus) (36).

The CpG motif has been proposed to act as a danger signal that warns of bacterial infection and activates immune defenses (37). Similarly, one function of therapeutic adjuvants may be to identify vaccine antigens as dangers to which the immune system should respond. Thus, a danger signal provided by CpG ODN may provide potent adjuvant function. Our studies demonstrate that CpG ODN are

extremely effective as adjuvants to induce Th1-dominated immune responses without significant toxicity. This property makes CpG ODN attractive as candidate adjuvants for potential use in human vaccines for the prevention or treatment of a wide range of infectious diseases and immune disorders.

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QS-21 structure/function studies: effect of acylation on adjuvant activity

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Abstract

QS-21 is a natural saponin adjuvant derived from the tree *Quillaja saponaria* Molina. Previous studies over a limited dose range suggested the acylation is critical to adjuvant activity. In this study, we prepared DS-1 (deacylated QS-21) and RDS-1 (reacylated DS-1 with dodecylamine at a different site than QS-21) to determine the effect on a dose-response curve over a wider range in mice. DS-1 and RDS-1 induced IgG1 responses at higher doses compared to that induced by QS-21. DS-1 was inactive for inducing IgG2a or CTL responses at any doses. RDS-1 showed moderate IgG2a response at 240 μg, but did not show CTL response at any dose evaluated.

Keywords: Adjuvant; QS-21; Structure/function; DS-1; GPI-0100

1. Introduction

QS-21 is a highly purified immunological adjuvant derived from the bark of the South American tree *Quillaja saponaria* Molina [1–4]. It is a water soluble triterpene glycoside with amphiphilic character that can be mixed with a soluble antigen resulting in a fully soluble vaccine formulation or that can be combined with emulsion or mineral salt adjuvants. This molecule promotes both humoral and cell-mediated immunity when added to parenteral or mucosal vaccine formulations [5–11]. QS-21 is currently under clinical evaluation for various vaccines [12] and has been tested in more than 3000 patients in 60 clinical trials. In recent clinical studies, QS-21 was shown to be more effective than aluminum hydroxide in stimulation of an antibody response to a malaria peptide vaccine [13] and to low dose HIV-1 gp120 [14]. QS-21 has also been used in clinical vaccines to induce cellular immune responses. A tyrosinase peptide vaccine containing QS-21 was shown to induce $CD8^+$ T-cells in a subset of patients in a metastatic melanoma clinical study [15].

In contrast to the majority of saponins from other species, *Quillaja* saponins are acylated. The three most predominant saponins (QS-17, QS-18 and QS-21) are acylated at the 4-hydroxyl position of fucose with two linked 3,5-dihydroxy-6-methyloctanoic acids containing a glycosylation site at the 5-OH position of one of the acyl chains. This acylation may be critical for adjuvant activity. Deacylated QS-18 and QS-21, each evaluated in mice at a 10 μg dose, were shown to induce a lower total IgG response to bovine serum albumin than that induced by the native acylated forms [2]. The deacylsaponin of QS-21 (termed DS-1) was evaluated as an adjuvant for antibody response (over the dose range of up to 40 μg) and CTL response (single dose of 10 μg) against ovalbumin in mice [16]. In contrast to QS-21, DS-1 did not stimulate a strong level of antibody (measured as total anti-OVA IgG) or OVA-specific CTL responses. The corresponding fatty acid fragment also appeared to be inactive [16]. Recently, Marciani et al. [17] reported on a preparation of semisynthetic triterpenoid saponins known as GPI-0100. GPI-0100 was prepared by deacylating a crude mixture of *Q. saponaria* saponins and then coupling dodecylamine with the carboxyl group of the glucuronic acid residue of the deacylated saponins through an amide bond. GPI-0100 was used as a reaction mixture without further purification. It was reported that GPI-0100 can stimulate a Th1 antibody isotype profile (IgG2a) as well as CTL production against exogenous antigens. A dose of >200 μg of GPI-0100 was equivalent to a dose of 10 μg of the corresponding native saponins for CTL response to OVA. The adjuvant activity of the deacylsaponin intermediates was not reported.

In this study, we compared the adjuvant activity of DS-1 (deacylated QS-21) and deacylated crude saponins (crude deacylsaponins) to QS-21 over a broad dose range. RDS-1 (HPLC purified GPI-0100 analogue of QS-21) and GPI-0100 were prepared and compared in an effort to understand the importance of acylation to the adjuvant activity of *Quillaja* saponins.

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2. Materials and methods

2.1. Synthesis of saponin analogues

Quillaja saponins were extracted from coarsely chopped *Q. saponaria* bark by previously described method [1]. *N,N'*-dicyclohexyl-carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dodecylamine and pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Ethyl acetate, acetonitrile and water were obtained from VWR Scientific (Boston, MA, USA). Triethylamine and trifluoroacetic acid (TFA) were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA) and Pierce (Rockford, IL, USA) respectively. All solvents were HPLC grade. Electrospray ionization (ESI) mass spectra were measured at Mass Consortium (San Diego, CA, USA). NMR spectra were recorded on a Varian-400 NMR Instrument at Jing Hong Custom NMR services (Cambridge, MA, USA). Saponin derivatives were analyzed by analytical high performance liquid chromatography (HPLC) by using a Vydac C4 column (4.6 mm × 25 cm, 5 μm particles) and eluted for 30 min with a 30–48% aqueous acetonitrile linear gradient in 0.15% TFA, followed by 15 min of 48% acetonitrile in 0.15% TFA. The flow rate was 1 ml/min and the effluent was monitored by an UV detector at 214 nm.

2.1.1. Preparation of DS-1 (deacylated QS-21)

DS-1 was prepared by deacylation of QS-21 as described previously [16].

2.1.2. Preparation of deacylated crude saponins (crude deacylsaponins)

Crude *Quillaja* saponins (1.0 g) were dissolved in 50 ml water, then 0.76 g triethylamine was added to adjust a final concentration of 0.15 M triethylamine (pH 12). After the mixture was stirred at 40–50 °C for 1 h, the reaction was terminated by adding acetic acid to adjust the pH to 7.0. The reaction mixture was then extracted with ethyl acetate and lyophilized. The final product was a white solid (0.82 g). The primary products of the reaction are DS-2 (deacylsaponin of QS-17 and QS-18) and DS-1 (deacylsaponin of QS-21).

2.1.3. Preparation of RDS-1 (reacylation of DS-1 and HPLC purification)

RDS-1 was prepared by mixing DS-1 (50 mg), dodecylamine (12 mg), NHS (7 mg), DCC (12 mg) and pyridine (1.0 ml). The reaction was stirred for 3 days at room temperature under N_2. Distilled water (0.1 ml) was added and the mixture was stirred for an additional 6 h to quench the reaction. Precipitated material was removed by centrifugation and then the mixture was evaporated on a rotary evaporator under vacuum to nearly dryness. The product was precipitated by adding 10 ml ethyl acetate and collected by centrifugation. The precipitate was washed twice with 2 ml ethyl acetate to afford a white solid (46 mg). ESI-MS (positive): *m/z* 1703 [*M* (DS-1 + dodecylamine) + Na]$^+$, 1742 [*M* (DCC adduct of DS-1 + DCC) + Na]$^+$, 1848 [*M* (diadduct of DS-1 + 2× dodecylamine) + H]$^+$, 1870 [*M* (diadduct of DS-1 + 2× dodecylamine) + Na]$^+$, 1887 [*M* (diadduct of DS-1 + dodecylamine + DCC) + H]$^+$, 1909 [*M* (diadduct of DS-1 + dodecylamine + DCC) + Na]$^+$.

A solution of 10 mg/ml of the above white solid in 30% aqueous acetonitrile containing 0.15% TFA was kept for 1.5–3 days at room temperature in order to allow all diadducts to decompose back to the aldehyde forms. Then 0.15 ml of the solution was injected into analytical HPLC and the peaks at 27–29.5 min and 33–38 min were collected separately (no TFA mobile phase was used). A total of five runs were carried out and the pools at 27–29.5 min and 33–38 min were combined, respectively. Acetonitrile was removed by rotary evaporation under vacuum at ~20 °C and the residues were dried by lyophilization to yield 0.8 mg DCC adduct product (27–29.5 min) and 4.2 mg RDS-1 (33–38 min) respectively. RDS-1 was obtained as a white solid. ESI-MS (positive): *m/z* 1703 [*M* + Na]$^+$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.45 (aldehyde), 1.20 (the dodecyl CH_2 groups). The DCC adduct product was also obtained as a white solid. ESI-MS (positive): *m/z* 1742 [*M* + Na]$^+$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.50 (aldehyde).

2.1.4. Preparation of GPI-0100 (reacylation of crude deacylsaponins)

The mixture of above deacylsaponins (100 mg), dodecylamine (24 mg), NHS (14 mg), DCC (24 mg) and pyridine (1.5 ml) was prepared and stirred for 3 days at room temperature under N_2. Then the reaction was quenched by adding 0.1 ml distilled water and stirred for an additional 6 h. After the precipitated material was removed by centrifugation, the mixture was evaporated on a rotary evaporator under vacuum to nearly dryness. The product was precipitated by adding 10 ml ethyl acetate and collected by centrifugation. The resulting white solid was washed twice with 2 ml ethyl acetate to yield crude GPI-0100 (67 mg). ESI-MS (positive): *m/z* 1703 [*M* (DS-1 + dodecylamine) + Na]$^+$, 1865 [*M* (DS-2 + dodecylamine) + Na]$^+$, 1742 [*M* (DS-1 + DCC) + Na]$^+$, 1904 [*M* (DS-2 + DCC) + Na]$^+$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.42 (aldehyde), 1.20 (the dodecyl CH_2 groups).

2.2. Immunization studies

2.2.1. Immunization protocol

Immunizations were carried out in C57BL/6 mice (female, 8–10-week-old at the time of the first immunization, 10 mice per group). The test vaccines consisted of 25 μg of the ovalbumin antigen (OVA, Grade VI, Sigma) and varying doses of the test adjuvant in a total volume of 0.2 ml phosphate buffered saline (PBS). Ovalbumin antigen (25 μg) itself without an adjuvant in a total volume of 0.2 ml PBS was used as a control and served as the "zero" adjuvant point for all groups. Vaccines were administered via the subcutaneous route and animals were immunized twice at a 2-week

interval (on days 0 and 14). Sera were collected 2 weeks after the second immunization for analysis by enzyme-linked immunosorbent assay (ELISA). Splenic mononuclear cells were collected 2 weeks after the last immunization for use as effector cells in the cytotoxic T-lymphocyte assay [9].

2.2.2. Immunological assays

Anti-OVA serum responses for IgG1 and IgG2a isotypes were determined by ELISA assays as described previously [5]. Cytotoxic T-lymphocyte responses, assayed as described previously [9], were measured as lysis of syngeneic target lymphoma cell lines (E.G7-OVA and EL4). The lytic effector cells were immunization-primed splenocytes, stimulated to mature to functional CTL by a 6-day in vitro culture with antigen (denatured OVA or mitomycin C-treated E.G7-OVA cells). Cytotoxicity was measured as lysis of ^{51}Cr-labeled target cells by splenocytes. The percent of ^{51}Cr release due to cytotoxicity was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release) where maximum release was measured after lysis of target cells with 1% NP-40 detergent and spontaneous release was measured after incubation of target cells with medium. The lysis of EL4 cells was subtracted from the lysis of E.G7-OVA cells to determine the percent antigen-specific cytotoxicity.

2.3. Hemolytic activity

An in vitro assay on red blood cells was used to screen QS-21 or analogues for hemolytic effects. Dulbecco's PBS (without calcium and magnesium) was dispensed in 100 μl aliquots to a 96-well U-bottom plate. QS-21 or analogues were serially diluted 1/2 into the buffer on the plate. Twenty-five microliters of sheep red blood cells (Biowhittaker), washed and diluted in PBS, were added to each well. The plates were incubated at room temperature for 30 min, centrifuged, 75 μl of the supernatants transferred to a flat-bottom 96-well plate and absorbance determined at 570 nm as a measure of released hemoglobin from the red cells. All analogues were assayed in triplicate. The concentration of QS-21 or analogues corresponding to 50% hemolysis was determined in each assay.

3. Results and discussion

3.1. Synthesis of saponin analogues

RDS-1 (GPI-0100 analogue of QS-21) and GPI-0100 were prepared by the literature method [17] from DS-1 and crude deacylsaponins, respectively. RDS-1 was purified by HPLC and GPI-0100 was used as a reaction mixture without further purification. Our results (based on MS analysis) showed that the reacylation reaction of DS-1 resulted in a mixture of four major products, RDS-1, a DCC adduct formed by coupling DCC with DS-1 and two diadducts (both the carboxy group and the aldehyde group were coupled with the hydrophobic chain). The primary GPI-0100 products consist of two amides by coupling dodecylamine with DS-1 (RDS-1) and DS-2 (RDS-2) as well as two amides by coupling DCC with deacylsaponins (DCC adducts) (for chemical structures, see Fig. 1, for HPLC profiles, see Fig. 2).

3.2. Stability and solubility of GPI-0100

Although GPI-0100 was soluble in PBS at 1 mg/ml, RDS-1 was poorly soluble in PBS and it could not be filter-sterilized. The imine forms and diadducts were not stable in solution. They decomposed back to the aldehyde forms immediately in PBS solution and slowly in 30% aqueous acetonitrile solution with or without 0.15% TFA (data not shown).

3.3. Immune responses

3.3.1. Antibody responses

The adjuvant effect of QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 were evaluated in C57BL/6 mice. All compounds were evaluated over a broad range of doses together with 25 μg OVA. All analogues were shown to stimulate a similar maximum IgG1 response to optimal dose QS-21 (Fig. 3). However, the minimum dose required for maximum stimulation for all compounds was increased by several fold compared to native QS-21. There was a larger dose-shift between the analogues for IgG2a response (Fig. 4). DS-1 did not induce IgG2a at any dose evaluated (up to 240 μg). The rank order for the other compounds for minimum effective dose was QS-21 (minimum effective dose for 10-fold increase = 10 μg) ≪ GPI-0100 = crude deacylsaponins (minimum dose for 10-fold increase ~160 μg) < RDS-1 (minimum dose for 10-fold increase ~240 μg). There was minimal difference for stimulation of antibody responses between GPI-0100 and crude deacylsaponins, as well as between RDS-1 and DS-1. This result indicated that addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly affect the adjuvant activity of these compounds for antibody response.

3.3.2. CTL response

DS-1 and RDS-1 elicit very low level CTL responses at all evaluated doses up to 240 μg. GPI-0100 and crude deacylsaponins can stimulate a very high level CTL response at high doses similar to optimal dose QS-21 (Fig. 5). Similar to antibody responses, there is no significant difference in the induction of CTL response between RDS-1 and DS-1, as well as between GPI-0100 and crude deacylsaponins. Therefore, addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly affect the adjuvant activity of these compounds for CTL response.

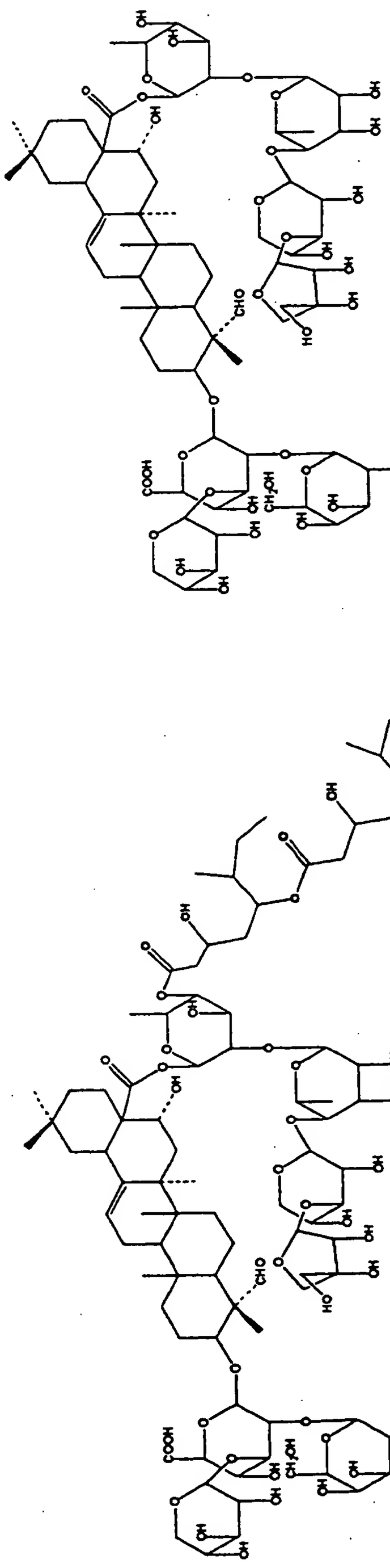

Fig. 1. Chemical structures of saponin derivatives.

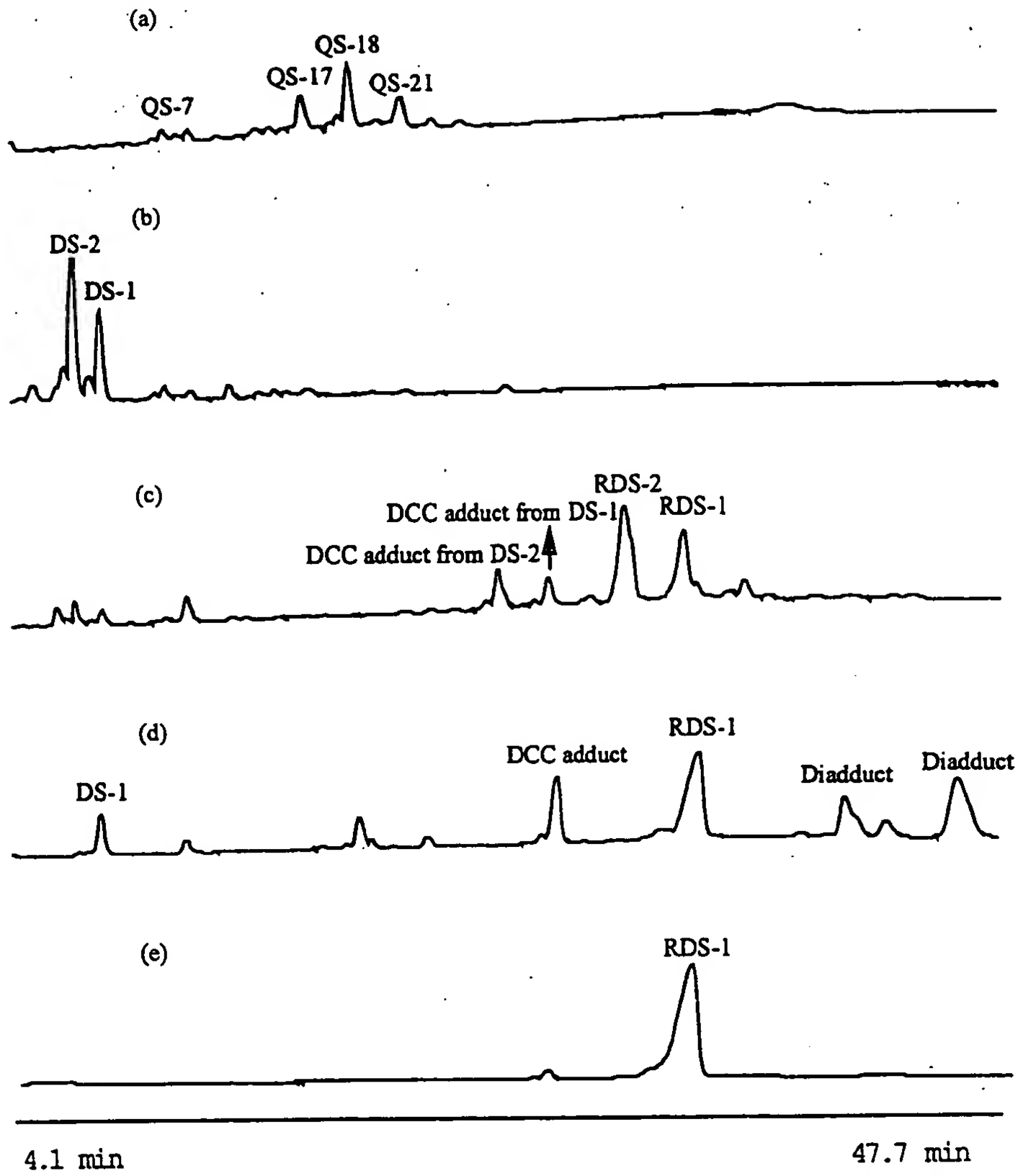

Fig. 2. HPLC profiles of (a) crude *Quillaja* saponins; (b) crude deacylsaponins; (c) GPI-0100; (d) reaction mixture of reacylation DS-1; (e) HPLC purified RDS-1.

Table 1
Fifty percent hemolysis by QS-21 or saponin derivatives (μg/ml)

QS-21	9.5
DS-1	93.0
Crude deacylsaponins	78.3
RDS-1	>500
GPI-0100	54.7

3.4. Hemolysis results

QS-21 is known to be a mild surfactant. One of the measures of this is a hemolytic assay on sheep red blood cells. This was compared between QS-21 and the analogues. RDS-1 was shown to be more than 100-fold less hemolytic than QS-21 as determined by the concentration required to produce 50% lysis. Similarly, DS-1 was shown to be less hemolytic than QS-21 by approximately 10-fold. The GPI-0100 and crude deacylsaponins were also shown to be several fold less hemolytic than QS-21 (Table 1). The dramatic decrease in hemolytic activity of RDS-1 may be due, in part, to its poor solubility.

3.5. Summary

DS-1, crude deacylsaponins, RDS-1 (HPLC purified GPI-0100 analogue of QS-21) and GPI-0100 were prepared and their immune adjuvant activities were evaluated. Similar to previous results, QS-21 was found to stimulate IgG1, IgG2a and CTL responses at doses of 10 μg or lower. DS-1 was compared over a wide dose range to determine the effect of the acyl chain on adjuvant activity. A previous evaluation of DS-1 suggested that DS-1 was generally inactive for induction of antibody responses at doses up to 40 μg [16]. In this study, DS-1 was shown to stimulate IgG1 response at high doses, but not IgG2a. The difference may be due to the measurement of a subset of IgG in this study (IgG1 isotype) rather than the measurement of total IgG in the previous study. The lack of activity for CTL response

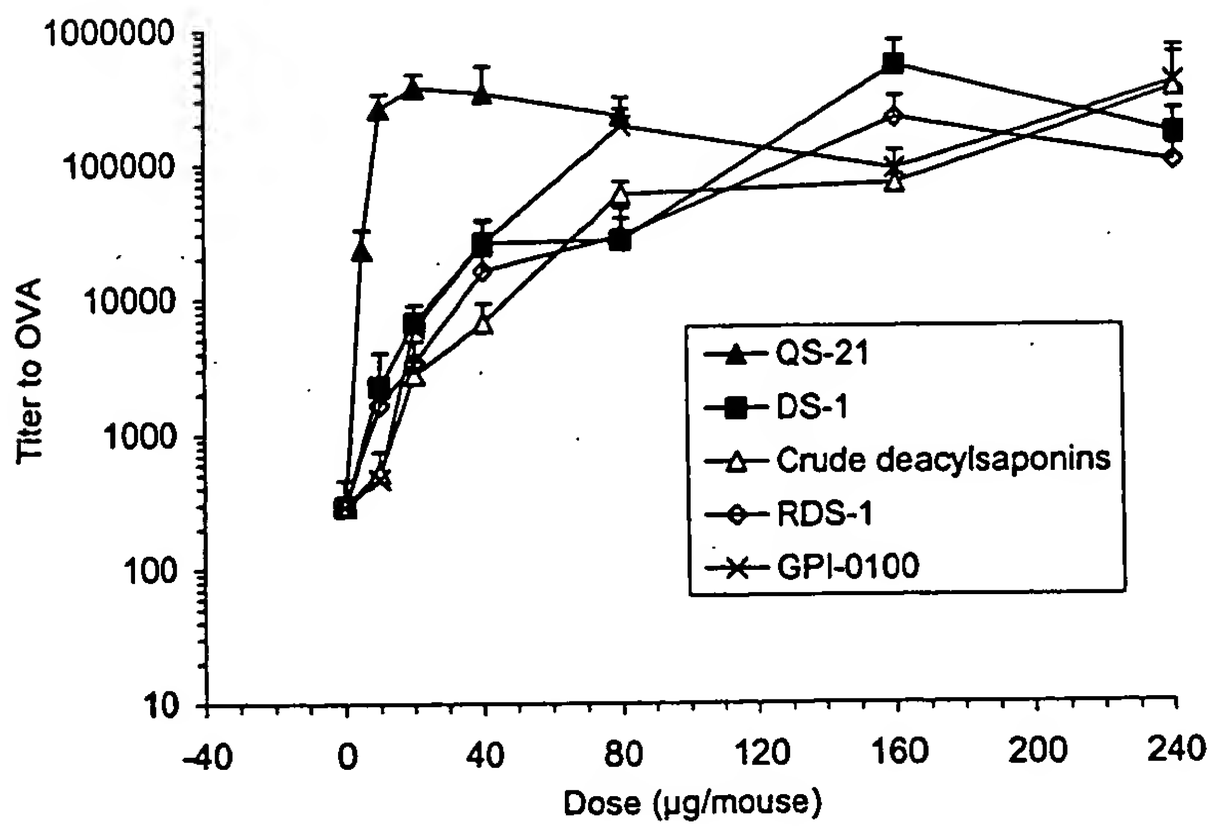

Fig. 3. Dose-response curves for QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 for stimulation of IgG1 to OVA. C57BL/6 mice (10 per group) were immunized by subcutaneous route at days 0 and 14 with 25 μg OVA and the indicated dose of adjuvant. OVA-specific IgG1 was determined by ELISA on pooled sera collected at day 28. Two-tailed Mann–Whitney statistical analysis indicated that all evaluated QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 dose groups are statistically significant compared to the ova control group ($P < 0.05$). If the analogues groups are compared with the equivalent dose QS-21 group, all are statistically significant ($P < 0.05$) except the comparison of 80 μg GPI-0100 group to 80 μg QS-21 group.

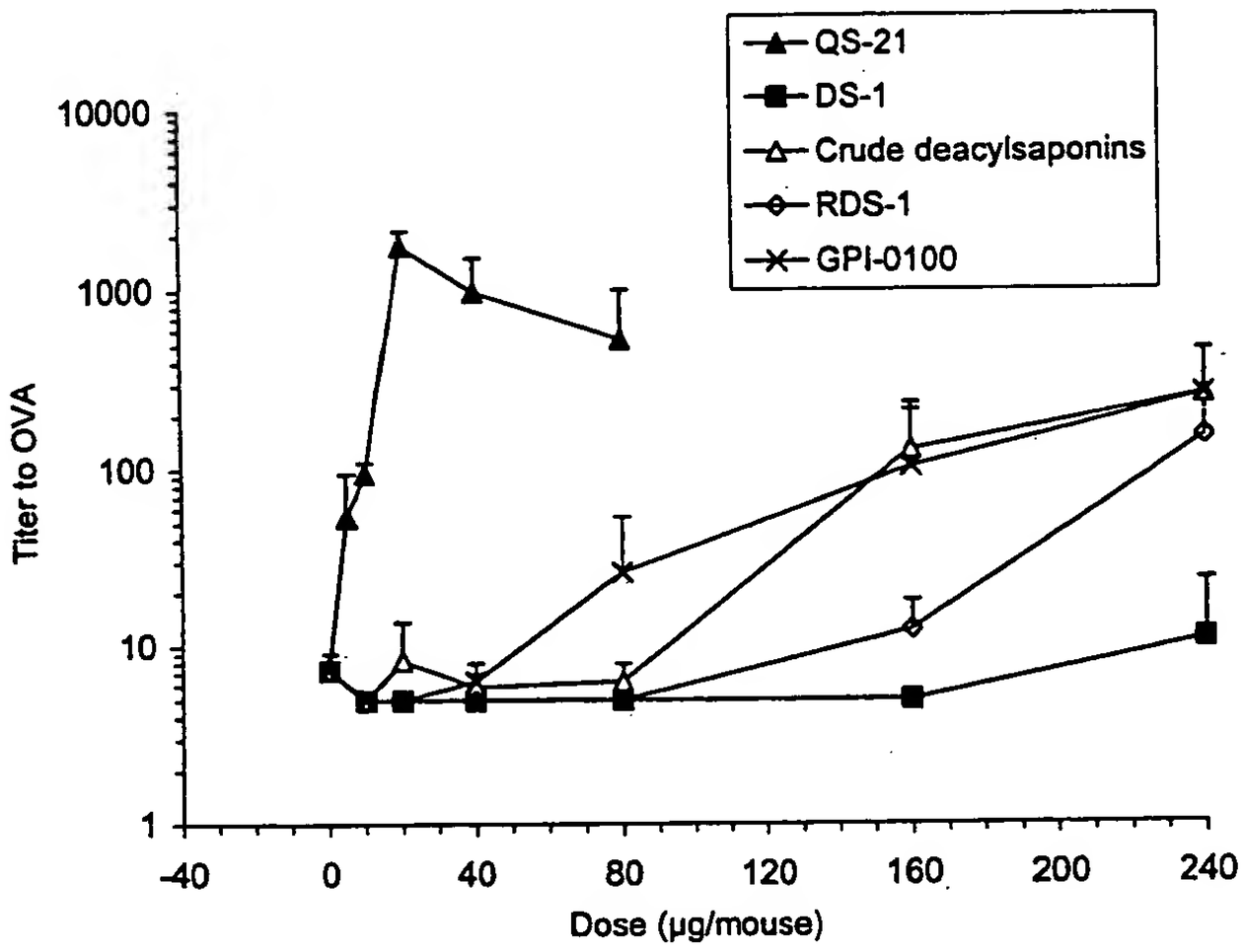

Fig. 4. Dose-response curves for effect of QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 on IgG2a. C57BL/6 mice (10 per group) were immunized by subcutaneous route on days 0 and 14 with 25 μg OVA and the indicated dose of adjuvant. OVA-specific IgG2a was determined by ELISA on pooled sera collected 2 weeks after the last immunization. Two-tailed Mann–Whitney statistical analysis indicated that all evaluated QS-21 dose groups are statistically significant compared to the ova control group ($P < 0.05$); all evaluated dose groups of DS-1 are not statistically significant compared to the OVA control group ($P > 0.05$); only the high dose crude deacylsaponins groups (160, 240 μg), the high dose RDS-1 group (240 μg) and the high dose GPI-0100 groups (160, 240 μg) are statistically significant compared to the OVA control group ($P < 0.05$). Compared to QS-21 (the same dose group comparison), all analogue groups are statistically significant ($P < 0.05$).

(measured previously only at 10 μg) was confirmed and found to extend to higher doses up to 240 μg. This suggests acylation is highly critical to Th1 type responses (CTL, IgG2a), but less critical to Th2 type responses (IgG1).

We also evaluated whether reacylation of DS-1 or crude deacylsaponins would restore the adjuvant effect for IgG1, IgG2a, or CTL associated with QS-21. Our results showed that: (1) QS-21 is a better adjuvant than RDS-1 and GPI-0100, especially for stimulation of IgG2a response; (2) there was no significant difference between GPI-0100 and crude deacylsaponins for stimulation of antibody (IgG1 and IgG2a) responses and CTL response. Similarly, there was

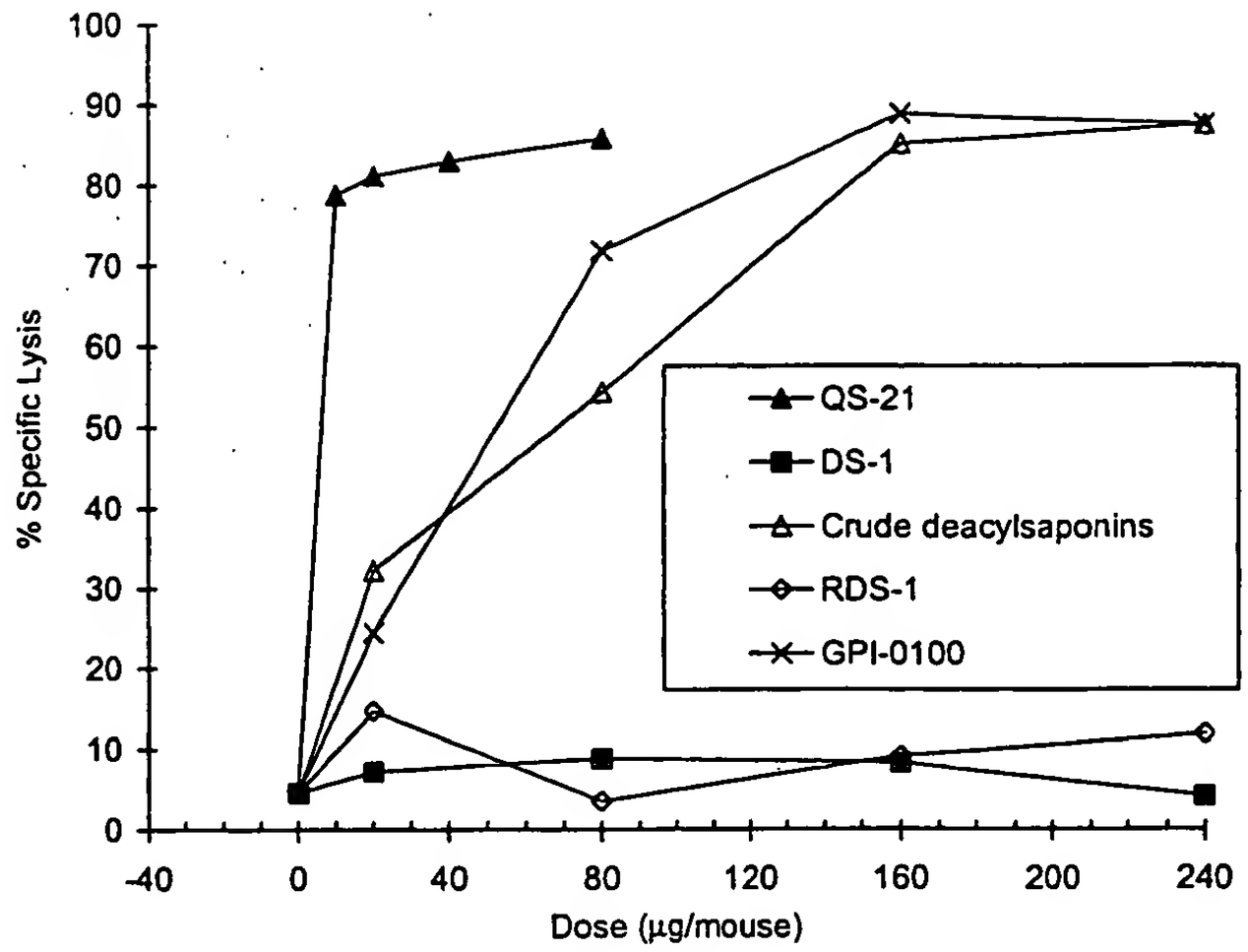

Fig. 5. Dose-response curves at 12:1 *E:T* ratio for production of antigen specific CTLs induced by QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100. C57BL16 mice (10 per group) were immunized by subcutaneous route on days 0 and 14 with 25 μg OVA and the indicated dose of adjuvant. The CTL response was measured on pools of splenocytes. Splenocytes were removed at day 28, expanded with antigen stimulation as described in Section 2 and used as effector cells. Lysis was measured against E.G7-OVA and EL4 cell targets.

no significant difference between the adjuvant activities of RDS-1 and DS-1. Therefore, addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly change the adjuvant activities. This suggests reacylation at a different site than the native compound with a synthetic fatty acid (dodecylamine) does not substantially improve the diminished adjuvant activity of the deacylated compound. We did not test other synthetic fatty acids, so cannot rule out a different result with a shorter or longer chain fatty acids. QS-7, an active native saponin, has a shorter acyl chain (C2). However, it is acylated at the original site on fucose [3]. Some residual IgG2a and CTL activity did remain in the crude deacylsaponins and GPI-0100. However, this may be due to lack of deacylation of QS-7 and other stable minor native saponins (Fig. 2). QS-7 is active in stimulating IgG2a and CTL responses [3]. It is also more stable than QS-21 under basic conditions (approximately 100-fold, Kensil, unpublished results). This is further supported by the lower activity of a reacylated saponin (RDS-1) prepared from a purified deacylsaponin (DS-1). This illustrates the importance of carrying out structure/function studies on purified natural products.

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SEPARATION AND CHARACTERIZATION OF SAPONINS WITH ADJUVANT ACTIVITY FROM *Quillaja saponaria* MOLINA CORTEX

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Saponins were purified from *Quillaja saponaria* Molina bark by silica and reverse phase chromatography. The resulting purified saponins were tested for adjuvant activity in mice. Several distinct saponins, designated QS-7, QS-17, QS-18, and QS-21, were demonstrated to boost antibody levels by 100-fold or more when used in mouse immunizations with the Ag BSA and beef liver cytochrome b_5. These purified saponins increased titers in all major IgG subclasses. To determine optimal dose in mice for adjuvant response, QS-7 and QS-21 were tested in a dose-response study in intradermal immunization with BSA in mice; for both of these purified saponins, adjuvant response (determined by stimulation of ELISA titers to BSA) neared maximum at doses of 5 μg and was shown to plateau up to the highest dose tested, 80 μg. These purified saponins vary considerably in their toxicity, as assessed by lethality in mice; the main component, QS-18, being the most toxic. Saponins QS-7 and QS-21 showed no or very low toxicity in mice, respectively. None of these saponins stimulated production of reaginic antibodies. The monosaccharide composition of these saponins showed similar but distinct compositions with all four containing fucose, xylose, galactose, and glucuronic acid. Predominant differences were observed in the quantities of rhamnose, arabinose, and glucose. Monomer m.w. (determined by size exclusion HPLC) were determined to range from 1800 to 2200.

Formulation of effective vaccines requires not only the appropriate Ag, but also the appropriate adjuvant to optimize protective humoral and cell-mediated immune responses. The use of the same Ag with different adjuvants has been shown to elicit significantly different responses from the immune system. For example, comparison of immunization of mice with killed schistosomula from *Schistosoma mansoni* with the adjuvants bacillus Calmette-Guérin, pertussis, *Coryne bacterium parvum*, tetanus toxoid, *Escherichia coli* LPS, yeast glucan, aluminum hydroxide, and saponin showed that only the animals immunized with bacillus Calmette-Guérin or saponin were protected from challenge (1) despite the demonstration of significant humoral immunity by some of the ineffective adjuvants. In effect, Allison et al. have noted that adjuvants such as aluminum hydroxide and mineral oil produce primarily humoral immunity whereas adjuvants such as muramyl dipeptide are able to induce cell-mediated immunity as well as differences in the isotype of the antibodies elicited (2). A further consideration, in addition to the efficacy of the adjuvant for eliciting a protective immune response, is the issue of toxicity of the adjuvant. CFA, which is used widely in research vaccines, produces excellent humoral and cell-mediated immunity, but is unsuitable for use in human and veterinary vaccines because of the toxic side effects (3). Similarly LPS, which is also a strong adjuvant, is highly toxic (reviewed in Reference 4). Hence, there is a need for identification of adjuvants that are both safe and efficacious.

One such potential adjuvant system is a class of compounds extracted from plant sources, termed collectively as saponins because of the detergent properties associated with them. The detergent properties of saponins are caused by their amphipathic nature; they consist of a hydrophilic carbohydrate moiety and a hydrophobic steroid or triterpene moiety. The adjuvant effect of saponins was noted in 1951 by Espinet (5) who utilized a crude saponin mixture to increase the immune response to foot-and-mouth disease vaccine. Extracts of the bark of a South American tree, *Quillaja saponaria* Molina, have been shown to be potent adjuvants (6–8). Further studies by Dalsgaard showed that adjuvant activity in these extracts resides in the saponin fraction, which has been characterized as a mixture of triterpene glycosides (7). Crude preparations of *Quillaja* saponins have been used to boost the response to BSA (7), keyhole limpet hemocyanin (9), SRBC (8), as well as aluminum hydroxide-based vaccines (9, 10). In addition, partially purified *Quillaja* saponins have been reported to associate with hydrophobic or amphipathic proteins and lipids to form detergent/lipid/saponin complexes termed ISCOM[4] (11); these structures are typically prepared by solubilizing the Ag with non-ionic detergents and then exchanging the non-ionic for the saponin detergent by centrifugation through sucrose gradients containing saponins at a concentration higher than their critical micellar concentration. ISCOM, which have been prepared from surface Ag isolated from influenza virus, measles, toxoplasma, feline leukemia virus, EBV, and HIV-1 (11–13) induce

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[4] Abbreviations used in this paper: ISCOM, immunostimulating complexes; MDP, muramyl dipeptide; MPL, monophosphoryl lipid A; TDM, trehalose dimycolate; TFA, trifluoroacetic acid; I.D., inside diameter.

serum antibody titers that are approximately 10-fold higher than immunization with protein micelles alone.

In addition to the potent adjuvant activity, the saponin fraction from *Quillaja* bark has strong hemolytic activity (7). This hemolytic activity has been suggested to be caused by the intercalation of saponins into cholesterol-containing membranes to form holes of approximately 80 Å, which can be observed with negative staining electron microscopy (14–16).

Despite the potential use of *Quillaja* saponins as adjuvants, their application has been limited because of the undesirable side effects of the commercially available preparations that are partially purified mixtures of saponins and other components (17–20). The adjuvant-active saponins have not been characterized because of the difficulty in purifying the active components to homogeneity. An adjuvant-active fraction was prepared from an aqueous extract of *Q. saponaria* bark by Dalsgaard (7) by using dialysis, anion exchange, and gel filtration chromatography in aqueous buffers; this fraction (designated Quil-A) was reported to be a single band by TLC on silica gel plates. However, we have found that this fraction is still a heterogeneous saponin mixture that can be resolved into multiple glycoside fractions by reverse phase HPLC. Higuchi et al. (21) have recently substantially purified a saponin from a methanolic extract of *Quillaja* bark and have characterized the glycoside moiety; however, this purified saponin was not tested for adjuvant effect. Hence, at present, there is no information on which components of the saponin fraction from *Quillaja* bark possess adjuvant activity. In this paper, we report a separation procedure for saponins extracted from the cortex of *Q. saponaria* Molina, identification of distinct saponin components with adjuvant activity and no apparent lethality in mice in an adjuvant-active dose range, identification of an adjuvant-saponin that is lethal at a lower dose than the original aqueous extract, and preliminary chemical characterization of these fractions.

MATERIALS AND METHODS

Purification of saponins. Coarsely chopped *Q. saponaria* bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h. The extract was centrifuged and the supernatant containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and the two supernatants were pooled. To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w/v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mM acetic acid with the hemolytic activity retained by the dialysis membrane.

The hemolytic fraction was lyophilized and redissolved at a concentration of 200 mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E. M. Science, Gibbston, NJ; 40 to 63 μm particle size, 2.5 cm I.D. × 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. The elution of saponins was monitored by carbohydrate assay (22). Fractions containing the saponins of interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in 50% methanol and loaded on a semipreparative HPLC column (Vydac C_4, 5 μm particle size, 3000 nm pore size, 10 mm I.D. × 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and individually rotavapped to dryness. Purity of saponins was assessed by analytic HPLC (Vydac C_4, 5 μm particle size, 3000 nm pore size, 4.6 mm I.D. × 25 cm length) with a gradient of 0.1% TFA in acetonitrile.

Immunologic procedures. CD-1 mice (8 to 10 wk of age) were immunized intradermally with a total volume of 0.2 ml injected at two sites per mouse. Each sample was tested in a group of five mice. The buffer used for all immunizations was PBS. The following proteins were used as Ag: BSA (Sigma) and purified cytochrome b_5 from beef liver, kindly provided by Dr. Philipp Strittmatter (University of Connecticut Health Center, Farmington, CT). CFA and IFA were obtained from Difco (Detroit, MI). MPL and TDM were obtained from Ribi Immunochemicals (Hamilton, MT). Squalene and Tween-20 were obtained from Sigma. Superfos Quil-A, a crudely enriched saponin preparation, and Alhydrogel (2% aluminum hydroxide) were obtained from Accurate Sciences, Westbury, NY.

The toxicities of Quil-A and purified saponins QS-7, 18, and 21, were tested in CD-1 mice by following procedures similar to those described above for immunizations. Varying doses of these compounds dissolved in sterile PBS were injected intradermally in mice. The mice were monitored for 72 h after injections and the results expressed in number of deaths per group.

Ag-specific antibody response was determined by ELISA. Immulon II plates were coated overnight at 4°C with 100 μl/well of coating solution, consisting of 10 μg/ml of the Ag in PBS. Plates were then washed twice with PBS and blocked in 10% normal goat serum (Hazelton, Rockville, MD) in PBS (150 μl/well for 1 h at room temperature). Plates were washed twice with 0.05% Tween 20 (Sigma) in water. Mouse serum was serially diluted 1/10 in 10% normal goat serum in PBS: 100 μl of each dilution was incubated on the plate for 1 h at room temperature. All dilutions were tested in duplicate on both Ag-coated and noncoated control wells. Plates were washed twice with 0.05% Tween 20. Goat anti-mouse IgG-horseradish peroxidase conjugate (H and L chain specific: Boehringer-Mannenheim Indianapolis, IN), diluted in 10% normal goat serum in PBS, was incubated on the plate (100 μl/well for 30 min at room temperature). The plates were washed four times with 0.05% Tween 20 and then with water two times. The substrate for the reaction was tetramethylbenzidine (23). Titers were determined from the dilution resulting in an absorbance of 0.5. Relative titers of specific antibody isotypes were determined by titration of sera pools (prepared with equivolume ratios of individual mouse serum samples in a group) on Ag-coated plates with the use of goat anti-mouse alkaline phosphatase conjugates specific for IgM, IgG3, IgG1, $IgG2_a$, and $IgG2_b$, respectively (Southern Biotechnology Associates, Birmingham, AL) and a goat anti-mouse IgE-horseradish peroxidase conjugate (Nordic, El Toro, CA).

Hemolytic activity. Serial 1/2 dilutions of saponin in PBS were made in a round bottom microtiter plate. The final volume in each well was 100 μl. SRBC (40% sheep blood and 60% Alsever's solution; Whittaker Bioproducts, Walkersville, MD) were washed three times by low speed centrifugation of the blood followed by resuspension of the red cell pellet in PBS to the original volume. The red cell pellet was diluted to 2.5 × the original volume and then used in the hemolysis assay. Twenty-five microliters of the resuspended cells were added to each well in the microtiter plate and mixed by pipetting. After incubation at room temperature for 30 min, the plates were spun at 1000 rpm for 5 min in a Sorvall RT6000 in an H-1000 rotor to sediment unhemolyzed cells. Fifty microliters of the supernatant from each well were transferred to the same well of a flat bottom microtiter plate. Absorbance caused by released hemoglobin was determined at 570 nm with a Dynatech microtiter plate reader.

Carbohydrate analysis. Relative carbohydrate concentration was determined by the anthrone method of Scott and Melvin (22). The standard for the assay was glucose. Analysis of carbohydrate composition as trimethylglucosides was carried out under contract by the Complex Carbohydrate Corporation (Athens, GA).

Monomer size of saponins. Monomer size of the saponins was determined by HPLC gel permeation chromatography on a Zorbax PSM 60 Si column (6.2 mm I.D. × 25 cm height). Ginsenoside Rb_1 (m.w. = 1109; Waco Pure Chemicals, Dallas, TX) and 18-β-glycyrrhetinic acid (m.w. = 471; Fluka Chemicals, Everett, WA) were used as m.w. standards. Saponins and standards were solubilized in methanol at a concentration of 1 mg/ml. Twenty microliters were injected on the column and eluted in methanol at a flow rate of 1.0 ml/min. Absorbance at 214 nm was used to monitor the column.

RESULTS

Isolation and characterization of saponin adjuvants. Approximately 20 to 25% of the dry weight of *Q. saponaria* Molina bark is extractable in water. Dialysis of the aqueous extract resulted in retention of approximately 24% of the dry weight and 95% of the hemolytic activity of the extract, indicating that saponins present in the

aqueous bark extract were retained by a dialysis membrane of 12,000 m.w. cutoff. Similar recoveries were achieved by chromatography of the aqueous extract on Sephadex G-50, with the saponin fraction localized in the void volume; reverse phase TLC showed that the identical components were isolated (not shown).

With the use of reverse phase HPLC, an unprocessed extract of *Q. saponaria* bark was shown to be a highly complex mixture. Treatment of this aqueous extract by ultrafiltration through a membrane with 10,000 m.w. cutoff removed almost all hydrophilic peaks from the retentate although multiple hydrophobic components were still present (Fig. 1*A*). Analysis of Quil-A, a commercial saponin that is commonly used in adjuvant studies, showed that this product contains all the peaks present in the ultrafiltrated aqueous bark extract shown in Figure 1*A*.

Significant resolution of the saponin peaks in the ultrafiltration retentate was achieved by using a shallow gradient of methanol or acetonitrile on Vydac C_4 as described in *Materials and Methods* (Fig. 1*A*). All major peaks in this retentate fraction were reactive with anthrone, indicating the presence of carbohydrate, and caused foaminess in aqueous solution, indicating that they were saponin in nature. Different bark samples yielded qualitatively a similar pattern of peaks with the same retention times. However, some quantitative differences were observed between different bark samples, apparently as a result of differences between the bark samples because extractions from the same sample of bark yield consistent results. The saponin peaks isolated by HPLC were tested for adjuvant activity by using BSA as the test Ag. Adjuvant-active components were identified in 10 of the peaks tested including the major peaks (7, 17, 18, and 21) (data not shown). These peaks, particularly peak 18, predominate in most samples of bark or commercial *Quillaja* saponins tested.

The major saponin peaks, purified as described in *Materials and Methods*, were further characterized for adjuvant activity as well as for physical and chemical properties. The purity of these samples is shown in Figure 1. The fractions, designated as saponins QS-7, 17, 18, and 21, with QS denoting the source to be *Q. saponaria*, are significantly pure in comparison with the starting extract, although several minor contaminants are evident in some fractions (Fig. 1 *B* to *E*).

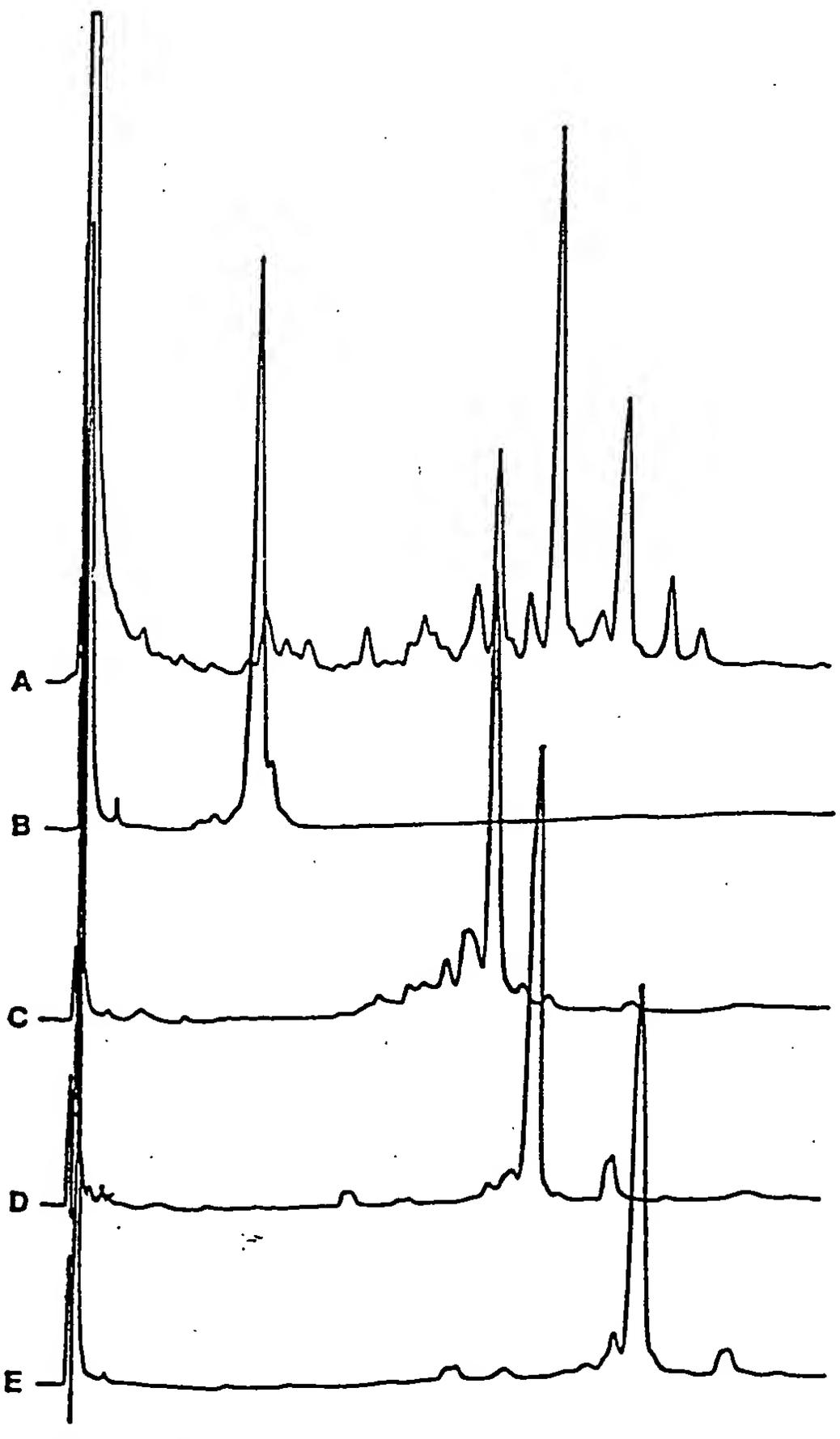

Figure 1. HPLC (Vydac C4, 4.6 mm × 25 cm, 5 μm particle size, 3000 nm pore size) of an aqueous bark extract treated by ultrafiltration (*A*), saponin QS-7 (*B*), saponin QS-17 (*C*), saponin QS-18 (*D*), and saponin QS-21 (*E*). Gradient was 30 to 40% 0.1% TFA/acetonitrile/30 min, 40%/15 min at a flow rate of 1 ml/min. A total of 100 μg of purified saponin or 200 μg bark extract (dry weight) was used per injection.

Effect of dose on adjuvant effect in mice. To establish the range of effectiveness for purified saponins, dose response curves were carried out for two of the saponins, QS-7 and QS-21 (Fig. 2). These saponins were chosen because they represented the most hydrophilic (QS-7) and hydrophobic (QS-21) of the four saponins purified in this study. Hydrophobicity was assumed to be related to the retention time on reverse phase HPLC with the use of a hydrophobic resin. CD-1 mice were immunized intradermally twice with BSA plus the indicated dose of saponin at 2-wk intervals. Sera was analyzed for anti-BSA IgG by ELISA 1 wk after the second immunization. Anti-BSA IgG titers were considerably augmented by doses of saponin as low as 5 μg for both QS-7 and QS-21. The immune responses obtained with QS-7 and QS-21 were similar, reaching a plateau at doses between 10 and 80 μg. No significant differences were observed between QS-7 and QS-21.

Adjuvant activity of purified saponins and research adjuvants. The purified *Quillaja* saponins (QS-7, 17, 18 and 21) were compared for effectiveness as adjuvants with various research adjuvants, such as aluminum hydroxide, CFA, and IFA, and a mixture of MPL and TDM. Saponins were used at a dose of 20 μg, an amount that falls in the plateau of maximum adjuvant effect observed with QS-7 and QS-21. Two immunizations with 10 μg of Ag cytochrome b_5 plus QS-7, 17, 18, or 21 in PBS resulted in an increase of approximately 10^3 in Ag-specific IgG ELISA titers when compared to a control group that received Ag alone. The titers observed in the groups receiving purified saponins were similar to those induced by the MPL/TDM mixture and CFA and IFA. However, purified saponins induced a higher response than aluminum hydroxide (Fig. 3).

Isotype of antibodies augmented by saponins. Adjuvants that augment similar IgG titers may differ considerably in boosting various IgG subclasses. Therefore, the

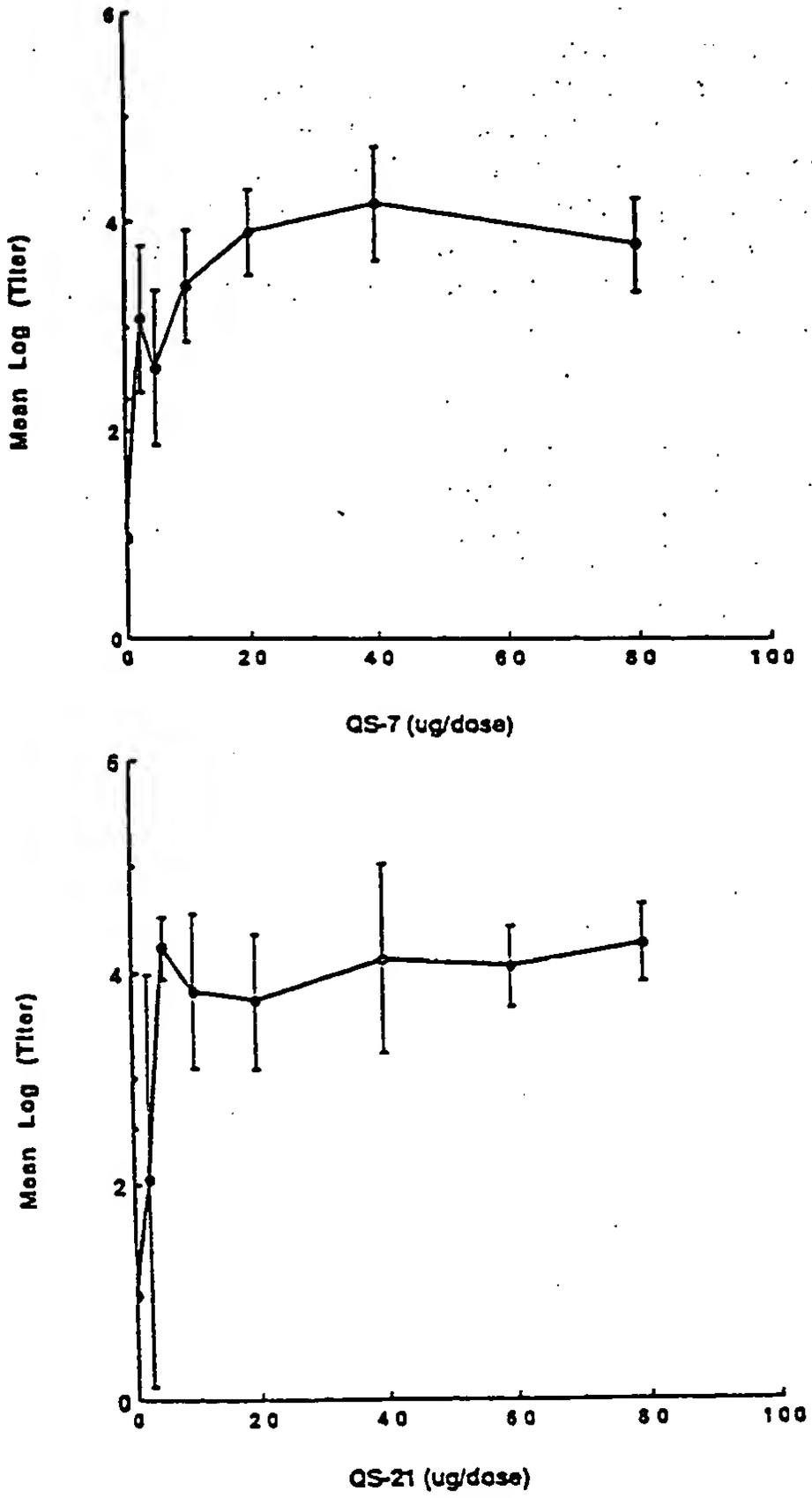

Figure 2. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 5 μg BSA and the indicated dose of QS-7 and QS-21. Results are expressed as means ± SD.

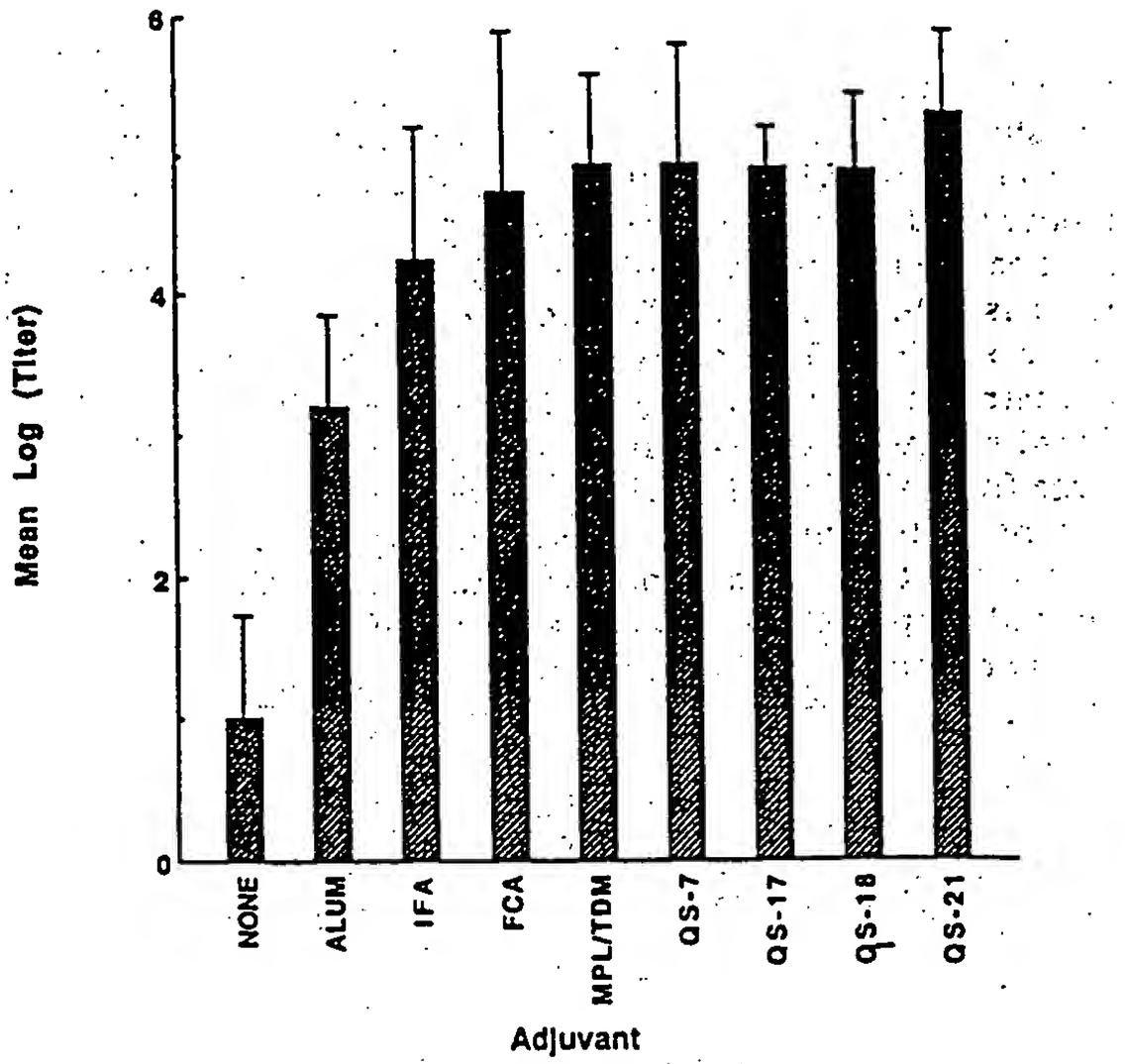

Figure 3. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 10 μg cytochrome b_5 and the indicated adjuvant. Formulations adjuvanted with CFA and IFA were prepared by emulsification of 100 μl of Difco CFA or IFA with 100 μl of a PBS/Ag solution/dose. MPL/TDM formulations were prepared by homogenization of 50 μg MPL, 50 μg TDM, 2 μl Squalene, and 0.2 ml 0.2% Tween 20/PBS/Ag/dose. The alum preparation contained 400 μg aluminum hydroxide per dose. The saponin preparations, which were fully soluble in aqueous solution, contained 20 μg of the indicated saponin in 0.2 ml PBS/Ag per dose. Results are expressed as means ± SD.

IgG subclass distribution of the IgG for the immunization experiment described in Figure 3 was determined. After two intradermal immunizations with cytochrome b_5 and saponins QS-7, 17, 18, and 21, antibodies were found in the three major IgG subclasses G1, $G2_b$, and $G2_a$ (Table I). With saponin fractions QS-17, 18 and 21, IgG2a antibodies predominated. In contrast, antibodies induced by Ag in PBS or on aluminum hydroxide were predominantly IgG1. CFA and MPL/TDM adjuvant augmented the production of isotypes IgG1, IgG2a, and IgG2b whereas IFA induced isotypes IgG1 and some IgG2b. In contrast to previous reports with the use of crude saponin preparations from *Q. saponaria* (2), no IgE antibodies were elicited by any of the purified saponins described here. Reaginic antibodies were not detectable at a 1/10 dilution for any of the adjuvants tested. The dose dependence of isotype distribution was not determined.

Purified saponins yielded consistent results in adjuvant effect. Five preparations of QS-21 that had been purified from different sources of *Q. saponaria* Molina bark were tested concurrently in an immunization study with BSA in mice; the mean and SD of the $\log_{10}$ ELISA titer of the five groups receiving three injections of 15 μg of QS-21 and 5 μg of BSA was 4.7 ± 0.13 in comparison with the control group which had a titer of 3.6.

TABLE I

Adjuvant effect on Ag-specific IgG Subclass

Adjuvant	Subclass Titer/Total IgG Titer[a]		
	G1	$G2_b$	$G2_a$
None	1.00	0	0
QS-7	0.35	0.21	0.44
QS-17	0.07	0.21	0.72
QS-18	0.10	0.06	0.84
QS-21	0.15	0.24	0.61
CFA	0.33	0.39	0.27
IFA	0.92	0.07	0.01
Aluminum hydroxide	0.91	0.09	0
MPL + TDM	0.24	0.38	0.38

[a] Sera were obtained at day 35 from the cytochrome b_5 immunization study described in Figure 3.

TABLE II

Lethality of saponins to CD-1 mice[a]

Dose (μg)	Quil-A	QS-7	QS-18	QS-21
125	1/5	0/5	4/5	0/5
250	2/5	0/5	5/5	0/5
500	4/5	0/5	5/5	1/5

[a] Results are expressed as number of deaths per group of five mice within 72 h after intradermal injection of saponins.

Toxic and hemolytic activities. Toxicity (assessed by lethality) has been associated with the use of saponins as adjuvants (20). In effect, the commercial saponin preparation Quil-A was lethal to mice in the dose range of 100 to 125 μg (Table II), as determined with one preparation. The purified saponins described here exhibit a wide range of lethalities. QS-18, the predominant saponin species in

the bark from *Q. saponaria* as well as in commercial preparations such as Quil-A, is the most lethal of those tested with deaths observed at doses as low as 25 μg (data not shown). In contrast, QS-7 is apparently nonlethal up to 500 μg and QS-21 is lethal only at 500 μg, with one mouse dead out of five mice receiving this dose (Table II). In mice, the minimum lethal dose/adjuvant-effective dose ratio is 50-fold for QS-21 and even higher for QS-7. However, the QS-18 adjuvant-effective dose is close to the lethal dose when assayed in mice. Apparently, the lethal effects of Quil-A can be explained in part by the large fraction of QS-18, which is the predominant component in its composition. The variability of QS-18 content in the bark used to prepare Quil-A and other commercial preparations will explain the differences in lethality observed with different preparations. From these results, we can state that there is no relationship between relative adjuvant activity and relative lethality.

The hemolytic activities of the purified adjuvant-saponins were compared. Saponins QS-17, 18, and 21 caused hemolysis of SRBC at concentrations as low as 5 to 30 μg/ml, with concentrations resulting in 50% hemolysis being 25 ± 0 μg/ml, 15 ± 3 μg/ml, and 7 ± 2 μg/ml, respectively (mean and SD of purified preparations derived from two separate bark samples). However, no hemolysis was observed with QS-7 at concentrations up to 200 μg/ml (highest concentration tested). There is no correlation between hemolytic activity, lethality and adjuvant activity, i.e., QS-7, 18 and 21, have approximately the same adjuvant activity but are widely different in hemolytic activity and lethality.

Carbohydrate composition. Purification of saponins allowed a preliminary structural characterization. The analysis of the composition of the four saponins QS-7, 17, 18, and 21 demonstrated the presence of a highly complex glycoside component, consisting of seven or more monosaccharides in saponin QS-7 and eight or nine monosaccharides in saponin QS-17 (Table III). All four saponins contained components with the same linkage, including terminal rhamnose, xylose, galactose, and glucose residues as well as 3-xylose, 2,3-glucuronic acid, and 3,4-rhamnose (linkage data not shown). It appears that these saponins share a common glycoside structure although there are clear deviations in the carbohydrate composition and linkage of the saponins analyzed.

All saponins contain arabinose except for saponin QS-7. Saponin QS-21 contains a diminished amount of glucose, suggesting that this may be caused by a contaminant as it is present in a ratio significantly lower than 1:1 when normalized to galactose. Monomer size of the predominant saponins was estimated by size exclusion HPLC. For comparison, we used triterpene and triterpene glycoside standards of known m.w. This analysis was carried out in methanol to prevent micellization. The monomer size ranges from 1800 to 2200 and is consistent with the m.w. predicted for a triterpene with 8 to 10 monosaccharide residues. It is likely that monosaccharides galactose, glucose, and glucuronic acid are each present in a ratio of 1.0 mol of monosaccharide/mol of saponin as higher molar ratios would significantly increase the m.w.

TABLE III

Molar ratio of monosaccharide/saponin[a]

Monosaccharide	Saponin			
	QS-7	QS-17	QS-18	QS-21
Rhamnose	2.22	2.34	1.15	1.27
Fucose	0.90	0.96	0.88	0.91
Arabinose	Trace	0.98	0.74	0.77
Xylose	1.28	1.33	1.34	1.44
Galactose	1.00	1.00	1.00	1.00
Glucose	1.35	1.23	1.16	0.35
Glucuronic acid	0.65	0.64	0.72	0.74

[a] Determined as trimethylsilated methyl glycosides and normalized to galactose (assumed to be present at 1 mol/mol of saponin).

DISCUSSION

These results demonstrate that the saponin fraction obtained by aqueous extraction of *Q. saponaria* bark is actually a heterogeneous group of related glycosides. All previous attempts to purify adjuvant-active *Quillaja* saponins have been in aqueous solution by methods typically used to purify proteins, such as dialysis, ion exchange chromatography, and size exclusion chromatography (7). Although these methods are useful in partially separating saponins from nonsaponin components, they have been ineffective in separating individual saponins because of the tendency of saponins to form mixed micelles. Hence, effective separation requires the use of organic solvents or solvent/water systems that solubilize the amphiphilic saponins as monomers so that the formation of mixed micelles does not interfere with separation. In effect, adsorption and reverse phase chromatography in organic solvents as described in *Materials and Methods* has allowed the purification of individual saponins to a degree of homogeneity that is significantly higher than that achieved by earlier reports (7, 24).

Although previous reports suggested that exposure to organic solvents destroyed adjuvant activity (25), we were able to recover adjuvant activity by using organic solvents for silica and reverse phase chromatography. The carbohydrate analysis of the individual saponins described in this paper indicate that they consist predominantly of one component, although some heterogeneity is still present because multiple linkage forms of individual monosaccharides can be detected. The carbohydrate composition and linkage analysis of the purified *Quillaja* saponins are similar to that determined by Higuchi et al. for the hydrolytic breakdown products isolated from a partially purified *Quillaja* saponin preparation (26). Dalsgaard reported that the saponin fraction isolated by anion exchange and gel filtration (Quil-A) contained the monosaccharides xylose, arabinose, glucose, rhamnose, and fructose (25) in unspecified ratios. None of the saponins described in this study contain fructose. In addition, they contain monosaccharide residues not reported by Dalsgaard (fucose, galactose, and glucuronic acid).

Adjuvant activity was demonstrated to be associated with several of the saponins, including those that appear to be most predominant, QS-7, 17, 18, and 21. Hence, the adjuvant activity of *Quillaja* bark extracts is associated with several distinct saponins rather than a single component, although the carbohydrate analysis indicates that these saponins may be structurally related. Not all peaks contained components that could serve as adjuvants in our test system.

Saponins QS-7, 17, 18, and 21 were tested more extensively because they were the predominant peaks in most bark samples analyzed. These fractions typically induced an increase in Ag-specific IgG titers when used at doses

ranging from 10 to 20 μg in intradermal immunization in mice. The adjuvant effect of these saponins was observed with both BSA and cytochrome b_5. Evidence that close proximity of Ag and saponin are important for the response was shown by our observation that saponin and BSA injected separately into different flanks of the mice did not induce a boost of Ag-specific IgG titers (data not shown), indicating no apparent systemic response. A similar result has been observed by Bomford (8). The strong antibody response elicited by ISCOM, which are reported to be a complex of saponin, Ag, and lipid (11, 12), are consistent with a close association of Ag and saponin being necessary for the adjuvant response. However, the adjuvant effects of saponins cannot be attributed simply to their detergent properties, i.e., saponin QS-7, which is a poor detergent as revealed by its nonhemolytic properties, has adjuvant characteristics similar to QS-17, 18, or 21, which are highly hemolytic.

Purified saponin adjuvants stimulate an equivalent or higher secondary immune response than that obtained by using aluminum hydroxide, CFA and IFA, or MPL/TDM adjuvants. ELISA titers measured via the end point dilution method, as was done in this study, are thought to be proportional to the concentration of high and medium avidity antibodies (27). Therefore, if it is assumed that the ELISA titers reported here reflect the concentration of these populations, then the purified saponins induce quantities of medium and high avidity IgG comparable with CFA, IFA, and MPL/TDM, and higher than those induced by aluminum hydroxide. However, differences in the concentrations of low avidity antibodies cannot be ruled out. Saponins also influence the Ag-specific isotype profile. A comparison of isotypes produced by mice in response to immunization with purified saponin showed induction of the three major IgG subclasses, G1, $G2_b$, and $G2_a$. The isotype profile observed with these purified saponins differs from that reported by Allison and Byars with a crude saponin (2) in which they found predominantly an IgG1 response to immunization of mice with Ag and crude saponin mixture, a response similar to that elicited by aluminum hydroxide. Under the immunization conditions utilized in this study, saponins induced significant levels of IgG2a and IgG2b as well as G1 antibodies; for some saponins, IgG2a predominated. Ag-specific IgE was not detected, even with the highly toxic QS-18, indicating that other components in crude preparations are responsible for the production of reaginic antibodies.

The high level of protection observed with the use of saponins with vaccines in mice (1) may in part be caused by the ability of saponins to induce an isotype profile similar to that observed in natural immunity arising from a viral or bacterial infection. Viral infections in mice induce an IgG response in which IgG2a accounts for 65 to 92% of total specific antibody (28). IgG2a has also been shown to be protective against protozoal infections (29). Both C fixation and antibody-dependent cellular cytotoxicity in mice can be mediated by IgG2a antibodies (30).

Commercially available saponin preparations are highly heterogeneous mixtures of adjuvant-active and inactive components. The relative concentrations of these components will vary according to the source of the bark, leading to difficulty in preparation of batches with a consistent composition. Substantial variation has been noted between different sources of commercially available saponins (31, 32). Purified saponins can be readily standardized, and this property allows preparation of vaccines with known proportions of a given active saponin or saponins.

The use of purified saponins for immunization allows selection of saponins with the optimal combination of adjuvant activity and negligible lethality. Preliminary studies indicate that some adjuvant-active saponins are significantly more lethal than others when tested at doses over the range of 25 to 500 μg in mice. It may be possible to select an adjuvant-active saponin for use in a vaccine that provides a wider safety margin between adjuvant-active and lethal doses than that in crude saponin extracts (which contains a larger fraction of lethal saponin adjuvants such as QS-18).

No attempt was made to correlate saponin structure with the biologic effects, adjuvant activity, and lethality associated with *Quillaja* saponins. A complete structural determination will involve sequencing of the glycoside moieties, identification of the triterpene, and identification of the point of linkage of the glycoside moieties onto the triterpene backbone. Comparison of the complete structures of naturally occurring variants such as those described here will provide information on what parts of the structure are involved in specific biologic activities. Further information on the minimal structure involved in these activities can be gained by analysis of less complex saponins produced by specific chemical or enzymatic hydrolysis of saponins of known structure. These studies are ongoing.

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Structure/Function Relationship in Adjuvants from *Quillaja saponaria* Molina

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A heterogeneous triterpene glycoside fraction from *Quillaja saponaria* Molina cortex has been utilized extensively as an immunological adjuvant, both in simple aqueous formulations (Dalsgaard 1974) and in the form of an immunostimulating complex (ISCOM) (Morein et al. 1984). Characterization of the structure of the adjuvant-active components in these extracts has been hindered by the heterogeneity of this fraction. We have purified the predominant triterpene glycoside adjuvants in crude *Quillaja* extract to near homogeneity by high-performance liquid chromatography (HPLC) (Kensil et al. 1991), allowing both structural and functional analyses. Three of these components, QS-17, QS-18, and QS-21, have been studied in more detail and form the basis of this study. Such an analysis is critical to the understanding of this important class of compounds, which have been shown to be potent adjuvants of both humoral and cell-mediated immune responses including class-I-restricted cytotoxic T lymphocytes (CTLs) to soluble proteins (Kensil et al. 1991; M.J. Newman, pers. comm.). Both types of immune responses are important to the efficacy of subunit vaccines against viral disease.

Comparative Structures of QS-17, QS-18, and QS-21

Figure 1 shows a proposed structure and the relationship among QS-17, QS-18, and QS-21. The basic structure is taken from Higuchi et al. (1988), who determined the structure of a compound (which he designated QSIII) from *Q. saponaria* that matches the carbohydrate composition and molecular weight of QS-17 reported here. We determined variations to this structure for QS-18 and QS-21 by carbohydrate composition and linkage analysis, molecular weights as determined by fast atom bombardment–mass spectroscopy (FAB-MS), and comparative analysis of common hydrolytic by-products (Fig. 1 and Table 1). The predominant changes in glycoside composition were in the terminal monosaccharides. QS-18 and QS-21 contained *t*-arabinose, whereas QS-17 contained 2-arabinose. QS-17 contained *t*-rhamnose (not present in QS-18 and QS-21), suggesting that *t*-rhamnose was linked to 2-arabinose in QS-17, whereas arabinose was a terminal residue in QS-18 and QS-21. In addition, QS-17 and QS-18 contained 3,4-rhamnose and *t*-glucose, whereas QS-21 contained 4-rhamnose and no glucose. This was indicative of glucose substitution at the 3 position of 3,4 rhamnose in QS-17 and QS-18.

Differences in the molecular weights, determined by FAB-MS, were consistent with these proposed structures. Further support was provided by comparison of hydrolytic by-products. Higuchi has demonstrated that mild alkaline hydrolysis of QSIII results in cleavage at the ester bond linking the fatty acid moiety to fucose. Cleavage of QS-17, QS-18, and QS-21 at this site should yield a triterpene glycoside fragment (A) that is identical for QS-17 and QS-18 and is more hydrophobic for QS-21 (due to absence of

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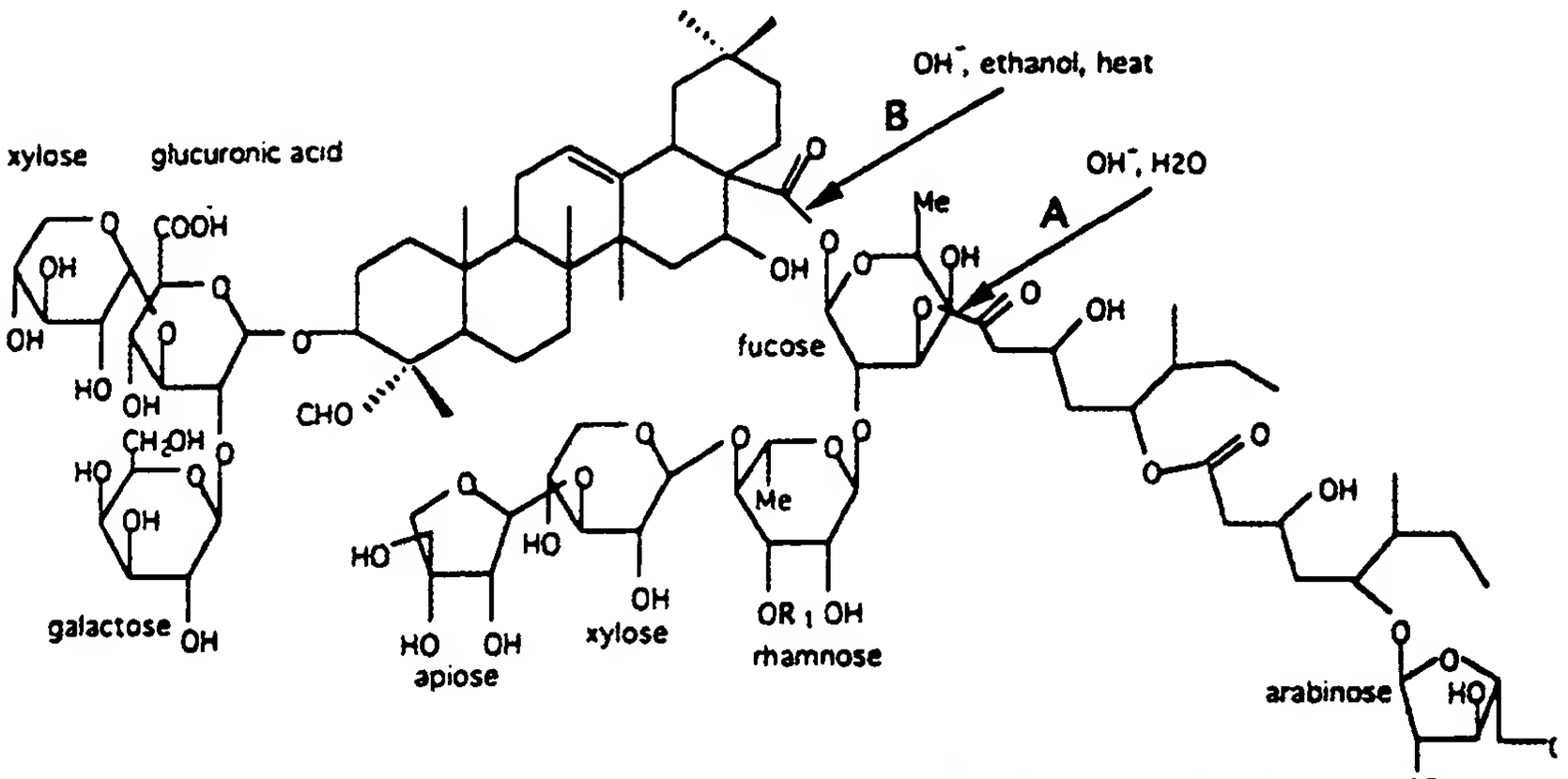

	R1	R2	m/z	Reverse Phase Retention Time(min) Fragment A	Fragment B
QS17	glucose	rhamnose	2321	8.0	26.7
QS18	glucose	H	2174	8.0	26.4
QS21	H	H	2012	9.3	25.6

Figure 1
Proposed structure of QS-17, QS-18, and QS-21. Structure of QS-17 was taken from QSIII (Higuci et al. 1988). Fragments A and B from each compound were generated by the indicated hydrolysi conditions, encompass the triterpene glycoside portion of the molecules, and were analyzed b reversed-phase HPLC.

Table 1
Carbohydrate Analysis of Purified *Quillaja saponaria* Adjuvants

	QS-17			*QS-18*			*QS-21*		
	AA[a]	*TMC*[b]	*linkage*	*AA*	*TMC*	*linkage*	*AA*	*TMC*	*linkage*
Rhamnose	184	2.34	T[c] 3,4	132	1.15	3,4[d]	132	1.27	4
Fucose	78	0.96	2	96	0.88	2	100	0.91	2
Arabinose	65	0.98	2	80	0.74	T	71	0.77	T
Xylose	82	1.33	T 3	118	1.34	T 3	114	1.44	T 3
Galactose	69	1.00	T	88	1.00	T	88	1.00	T
Glucose	86	1.23	T	89	1.16	T	–	–	–
Glucuronic acid	n.t.[e]	0.64	2,3	n.t.	0.72	2,3	n.t.	0.74	2,3
Apiose	24.5[f]	n.t.	T	25.7	n.t.	T	20.0	n.t.	T

[a]Alditol acetate (μg/mg compound).
[b]Trimethylsilated methyl glycosides (mole monosaccharide/mole galactose).
[c]Terminal glycosyl residue.
[d]Trace amounts of T-rhamnose also detected.
[e]n.t. indicates not tested.
[f]Poor recovery as alditol acetates.

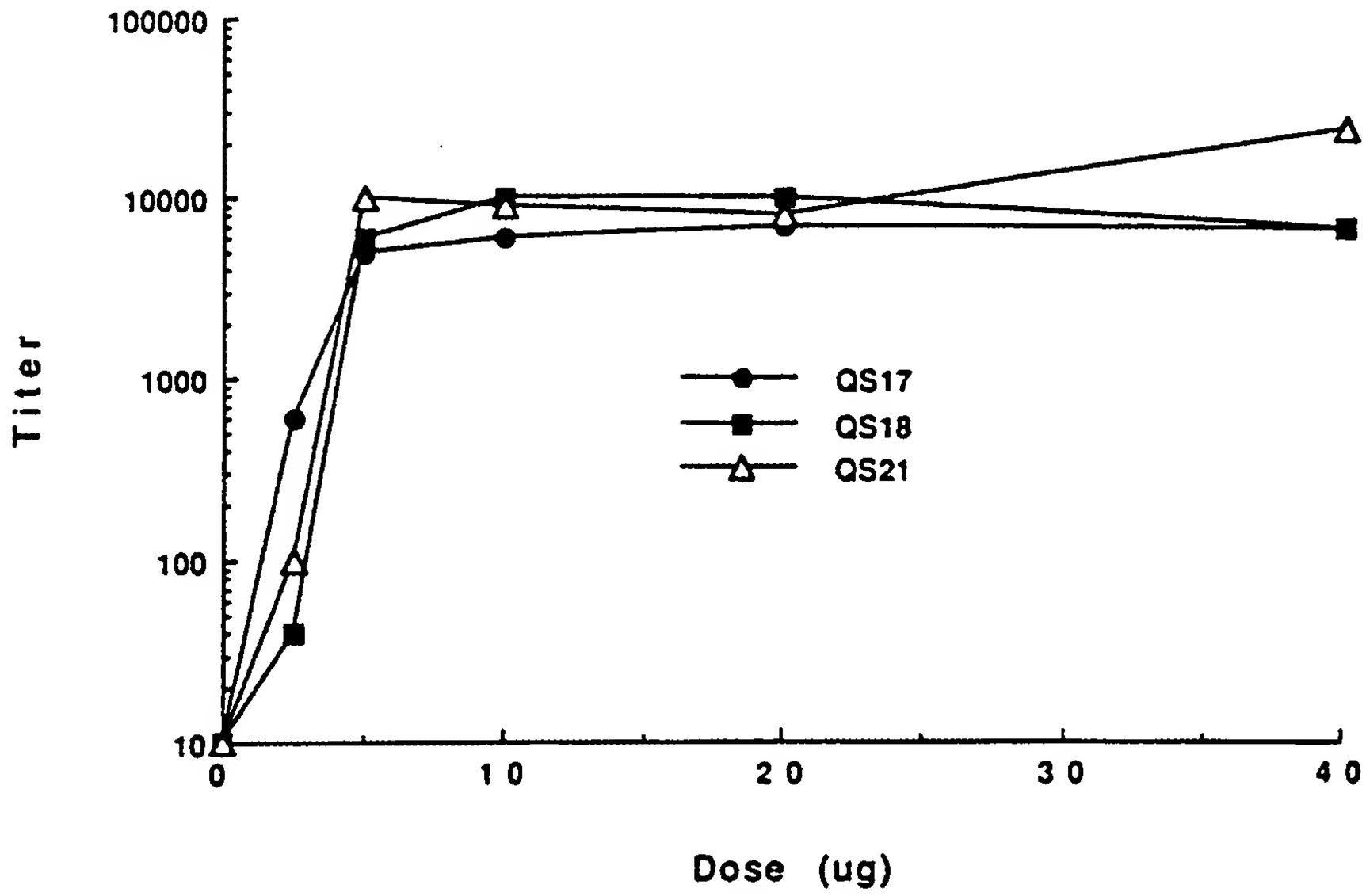

Figure 2
Dose response study of QS-17, QS-18, and QS-21 for antibody stimulation. CD-1 mice (five per group) were immunized intradermally with 5 μg of BSA and the indicated dose of adjuvant at day 0 and day 14. Sera were analyzed by EIA on BSA-coated plates on day 21.

glucose); this was confirmed experimentally by reversed-phase HPLC retention times of the fragments from these compounds (Fig. 1). These compounds were hydrolyzed under more severe conditions to cleave the ester bond linking fucose to the quillaic acid backbone; the limiting triterpene glycoside fragment (B) resulting from this cleavage should be identical for all three compounds. This was confirmed by HPLC analysis (Fig. 1).

All three compounds, QS-17, QS-18, and QS-21, had been shown to augment humoral immune responses in mice with similar dose response curves (Fig. 2). Hence, it would appear that the terminal residues rhamnose and glucose are not critical to this facet of the adjuvant function of these compounds.

Influence of Structural Modifications on Adjuvant Activity

Modification of these compounds was carried out to determine the effect on antibody stimulation. QS-18 was modified by periodate oxidation, which preferentially generates aldehydes from *cis* vicinal hydroxyl groups. Hence, *t*-galactose and *t*-apiose were likely targets of this reagent, allowing assessment of the importance of these monosaccharides to adjuvant function. The periodate-oxidized QS-18 was tested for augmentation of antibody response to bovine serum albumin (BSA) in mice (Fig. 3A). Periodate oxidation of QS-18 eliminated adjuvant activity. Hence, either galactose or apiose (or both), which are monosaccharides common to all known adjuvant-active compounds from *Q. saponaria* for which carbohydrate composition data are available, appears to be essential for antibody stimulation.

Hydrolytic by-products of QS-18 and QS-21 (equivalent to fragment A of Fig. 1) were prepared to determine the effect of removal of the fatty acids and arabinose on adjuvant

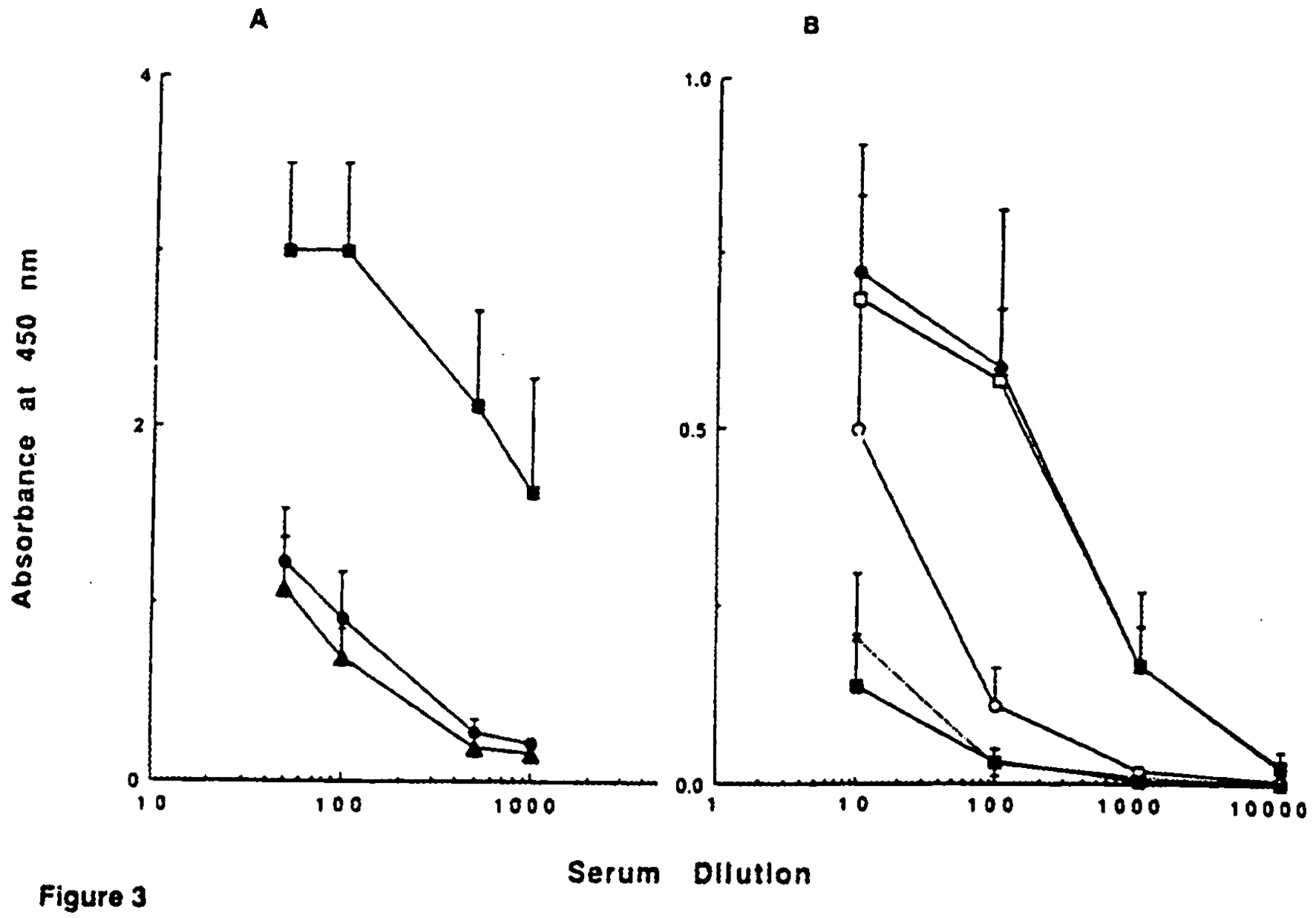

Figure 3
(*A*) Comparison of adjuvant effect of QS-18 and periodate-oxidized QS-18. (*Closed circle*) No adjuvant; (*closed triangle*) periodate oxidized QS-18; (*closed box*) QS-18. (*B*) Comparison of adjuvant effect of QS-18 and QS-21 with the respective fragment A. (*Dotted line*) No adjuvant; (*open box*) QS-18; (*closed box*) fragment A of QS-18; (*closed circle*) QS-21; (*open circle*) fragment A of QS-21. CD-1 mice (3–4 per group) were immunized intradermally with 10 μg BSA and 10 μg of the indicated adjuvant. Sera were analyzed by EIA on BSA-coated plates on day 14.

function. QS-18 and QS-21 were treated by mild alkaline hydrolysis to generate their respective fragment A. These fragments were substantially more hydrophilic than the original compounds due to loss of the fatty acids and arabinose. These fragments were tested in mice for induction of antibody to BSA (Fig. 3B). The fatty-acid-free fragments induced a substantially lower antibody response than the formulations containing the intact adjuvants. We had previously suggested that a close association of antigen and *Q. saponaria* adjuvants could be important for optimum immune response (Kensil et al. 1991). We have observed that adjuvant injected in a site different from that of antigen is ineffective. QS-18 and QS-21 have been shown to bind to BSA (data not shown). One possible mechanism for this decreased antibody stimulation by the fatty-acid-free glycosides is that the binding to the antigen through hydrophobic interactions is reduced or eliminated due to the absence of the fatty acid. However, it should be noted that the loss of adjuvant function of periodate-oxidized QS-18 could not be attributed to change in hydrophobicity of the compound because the oxidation had only a minimal effect on hydrophobicity.

To investigate the importance of the close association further, we have covalently coupled QS-21 to a protein antigen, hen egg lysozyme. Lysozyme was chosen for this experiment because in general it is poorly immunogenic, and being a hydrophilic protein, it is unlikely to bind to QS-21 through hydrophobic interactions. A conjugate (1:1 molar ratio of QS-21:lysozyme, prepared by coupling the carboxylic acid on QS-21 glucuronic acid to protein amino groups with carbodiimide chemistry) was tested in C57BL/6 mice (Fig. 4). QS-21 failed to produce detectable anti-lysozyme antibody titers in mice im-

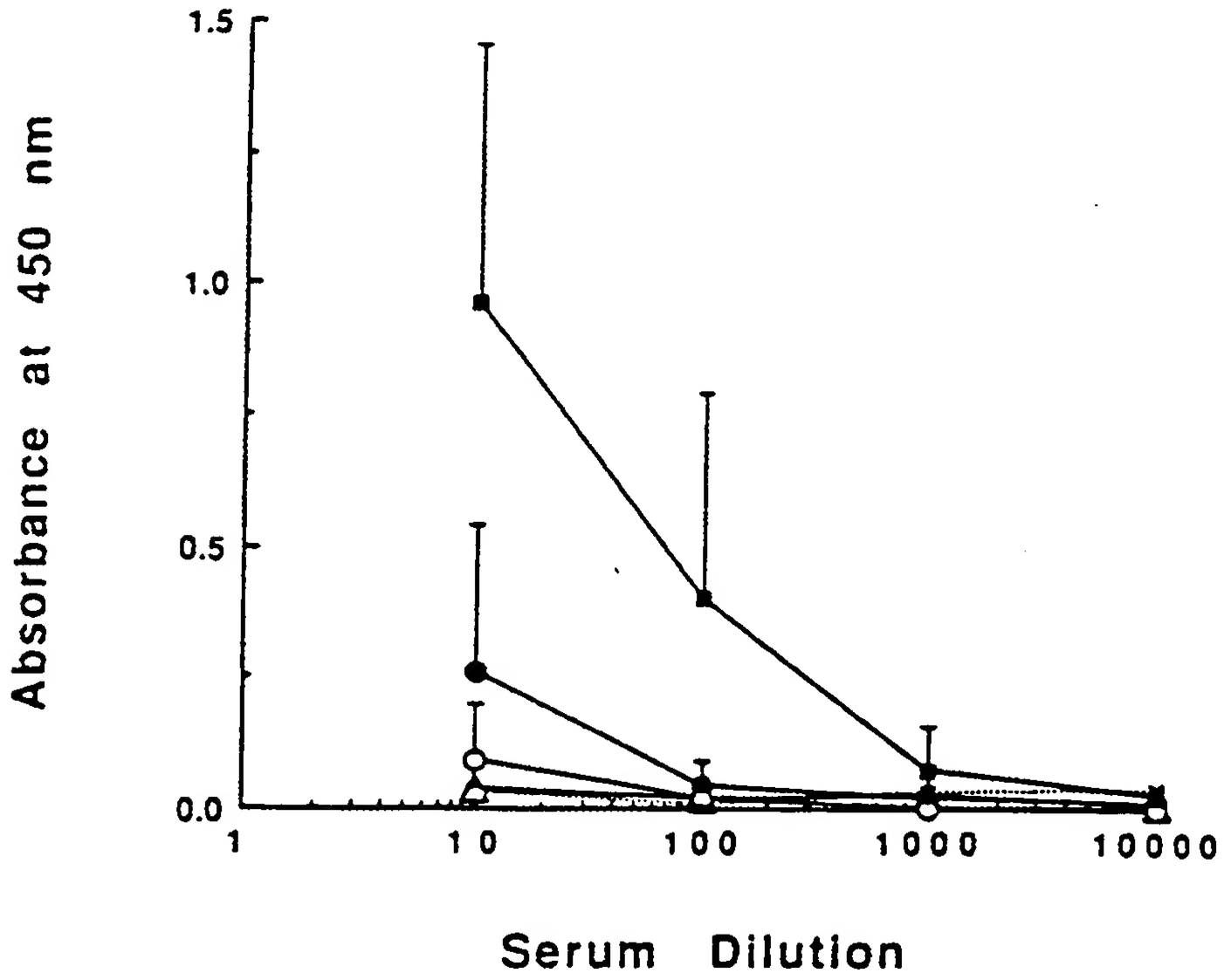

Figure 4
Comparison of immune response of an antigen-QS-21 conjugate compared to unconjugated antigen and QS-21. C57BL/6 mice (ten per group) were immunized intradermally at 1 and 14 days with lysozyme or lysozyme-QS-21 conjugates. Sera were analyzed by EIA on lysozyme-coated plates on day 21. (*Dotted line*) 10 μg lysozyme; (*open triangle*) 10 μg lysozyme/1.6 μg QS-21 (noncovalent); (*closed circle*) 10 μg lysozyme/1.6 μg QS-21 (covalent); (*open circle*) 10 μg lysozyme/10 μg QS-21 (noncovalent); (*closed box*) 10 μg lysozyme/1.6 μg QS-21 (covalent) + 10 μg QS-21 (noncovalent).

munized twice with 1.6 μg of free QS-21 mixed with lysozyme and only increased titers slightly in mice immunized with 10 μg of free QS-21, a result that we think may be due to lack of QS-21 binding to lysozyme. Mice immunized with an equimolar conjugate (amount of bound QS-21 = 1.6 μg) produced an immune response to lysozyme that exceeded that even of mice receiving 10 μg of QS-21 in noncovalent form. Addition of 10 μg of free QS-21 to the conjugate induced the highest responses, suggesting that the covalently attached QS-21 served as a association site for an additional one to two molecules of QS-21 to lysozyme.

SUMMARY

We have shown that galactose and apiose, monosaccharides common to the structures of QS-17, QS-18, and QS-21, are critical to their function in stimulation of antibody. In contrast, *t*-rhamnose and *t*-glucose do not seem to be important because these monosaccharides are the primary points of deviation among these three compounds, which are very similar in antibody stimulation. The fatty acid region also appears to play an important role. In addition, the data on conjugation of QS-21 to antigen suggested that close association of antigen and this adjuvant is important. At present, little is known about the mechanism of this important class of adjuvants. Future studies will entail determination of the cellular site of action and development of in vitro assays to assess the function of this class of important compounds.

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STRUCTURE OF DESACYLSAPONINS OBTAINED FROM THE BARK OF *QUILLAJA SAPONARIA*

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Key Word Index—*Quillaja saponaria*; Rosaceae; quillaja bark; quillajasaponin; triterpenoid saponin; desacylsaponin; diazomethane degradation; quillaic acid 3,28-*O*-bisglycoside; quillaic acid.

Abstracts—A triterpenoid saponin mixture (so-called quillajasaponin) obtained from the bark of *Quillaja saponaria* was treated with weak alkali and two major desacylsaponins were isolated. On the basis of chemical and spectral evidence, they were determined as 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-[*β*-D-xylopyranosyl-(1 → 3)]-*β*-D-glucuronopyranosyl quillaic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-fucopyranoside and 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-glucopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-fucopyranoside. Diazomethane degradation providing selectively the 28-*O*-glycoside from the 3,28-*O*-bisglycoside was a useful method for the structure elucidation.

INTRODUCTION

The bark of *Quillaja saponaria* Molina, named quillaja bark (cortex quillajae), is known as a saponin crude drug and has been used as a detergent, dentifrice and expectorant [1]. The existence of a saponin mixture (designated as quillajasaponin), which was recently reported to have a strong adjuvant activity [2, 3] and a plasma cholesterol lowering effect [4], was recognized but as for the constituents of the crude saponin, little was known except for quillaic acid [5, 6] and its monoglucuronide [7], which were obtained upon acid hydrolysis of the saponin. A study on the constituents of the quillajasaponin has been conducted in an attempt to isolate the physiologically active triterpenoid compounds. We report in this paper the structures of two desacylsaponins (quillaic acid 3,28-*O*-bisglycosides), DS-1 (1) and DS-2 (2), obtained by mild alkaline hydrolysis of the quillajasaponin.

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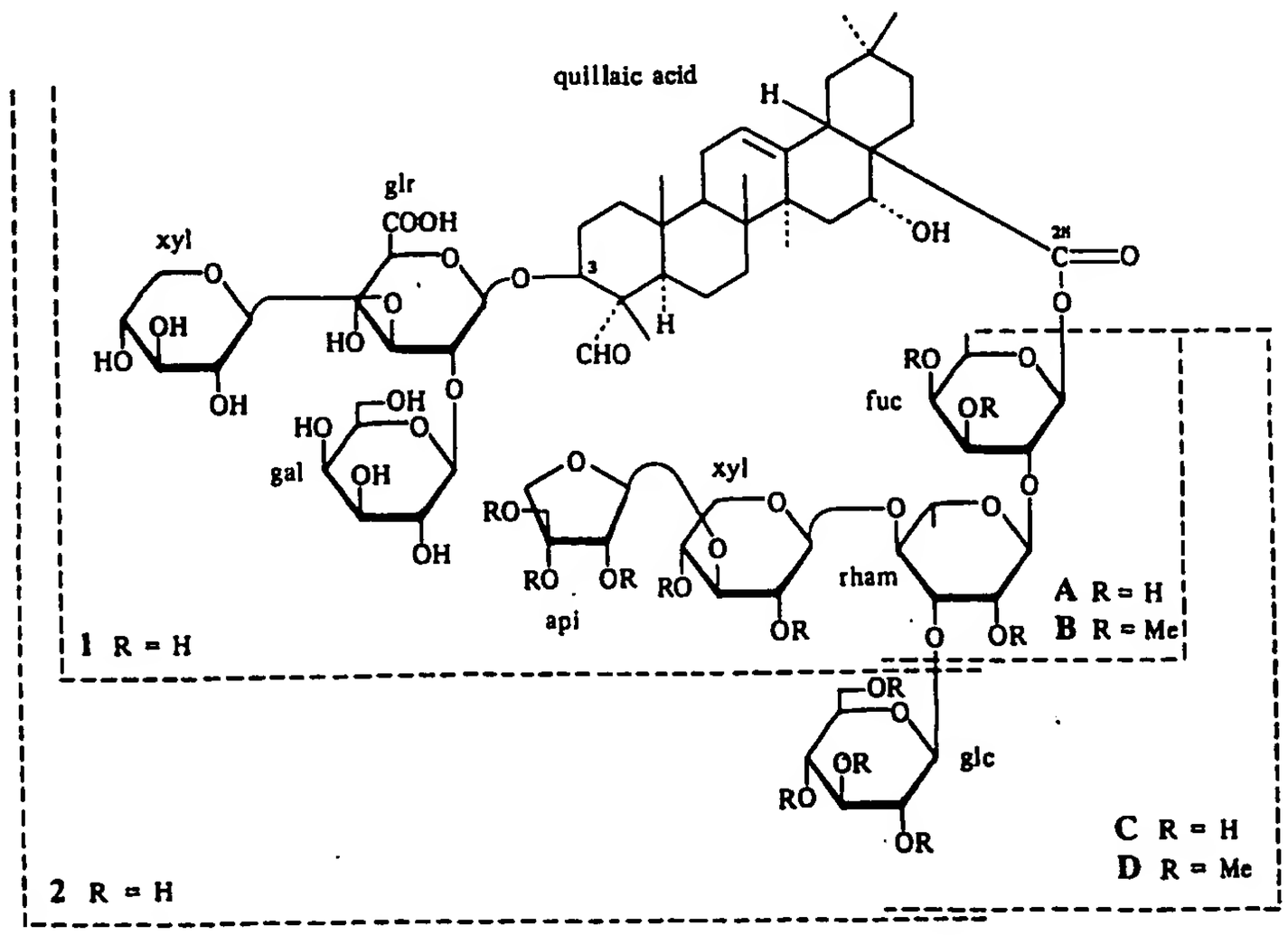


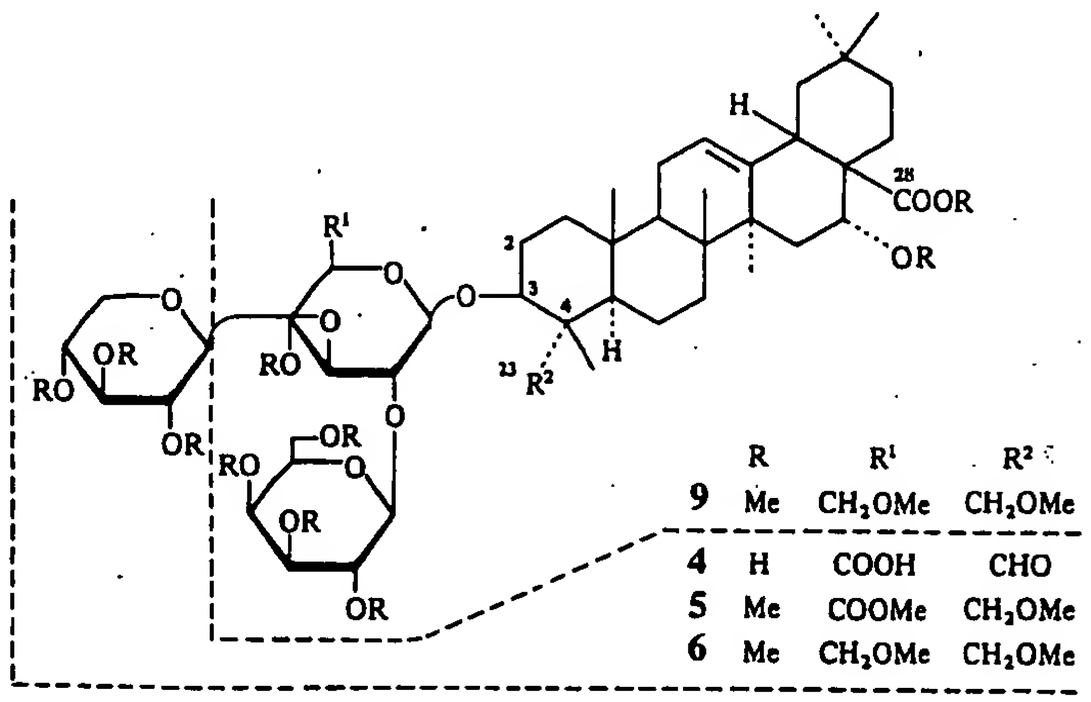
RESULTS AND DISCUSSION

The methanol extract of the bark was fractionated by the ordinary procedure, as described in the Experimental, to give a saponin fraction (quillajasaponin). On treatment with 6% $NaHCO_3$ in 50% methanol, the fraction afforded a desacylsaponin mixture, which was separated by normal and reverse phase column chromatography to give two major compounds, DS-1 (**1**) and DS-2 (**2**). Compound **1**, as well as **2**, showed a single spot on normal and reverse phase TLC, respectively.

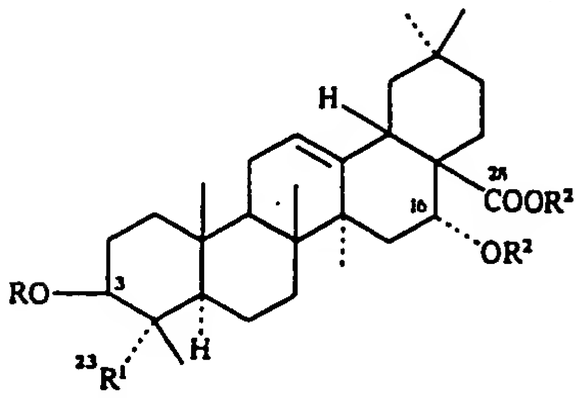
Compound **1** was hydrolysed with acid to yield glucuronic acid (Glr), galactose (Gal), xylose (Xyl), fucose (Fuc), rhamnose (Rham), apiose (Api) and quillaic acid (**3**). Compound **2** yielded the same products as those of **1** and in addition glucose (Glc). The ^{13}C NMR spectra of **1** and **2** showed seven and eight anomeric carbon signals respectively. The FAB mass spectra of **1** and **2** revealed the molecular ion peaks as a cationized cluster ion at *m/z* 1589 $[M+2K-H]^+$ and 1751 $[M+2K-H]^+$, respectively. These data indicate **1** to consist of 1 mol each of **3**, Glr, Gal, Xyl, Fuc, Rham, Api and another one, Xyl or Api, and **2** to consist of the same components as those of **1** and 1 mol of Glc. Both compounds were suggested to have a 28-*O*-glycosidic linkage since in their ^{13}C NMR spectra the signals due to C-28 of the aglycone part were observed at δ176.0 (in **1**) and 176.3 (in **2**) [8].

When compounds **1** and **2** were treated with 2% potassium hydroxide in 50% ethanol, they afforded the same prosapogenin (**4**), which was hydrolysed with acid to yield **3**, Glr, Gal and Xyl. Compound **4** showed in the ^{13}C NMR spectrum three anomeric carbon signals and three carbon signals at δ24.6, 54.9 and 84.2 due to C-2, C-4 and C-3 of quillaic acid having a sugar moiety at its 3-position [9], and showed the molecular ion peak at *m/z* 995 $[M+K]^+$ in the FAB mass spectrum. These data indicate that **4** is the 3-*O*-glycoside of **3** and consists of 1 mol each of **3**, Glr, Gal and Xyl. Since the aldehyde group and Glr unit in **4** were thought to complicate its structure elucidation, compound **4** was converted to the more stable compound **6** as follows.

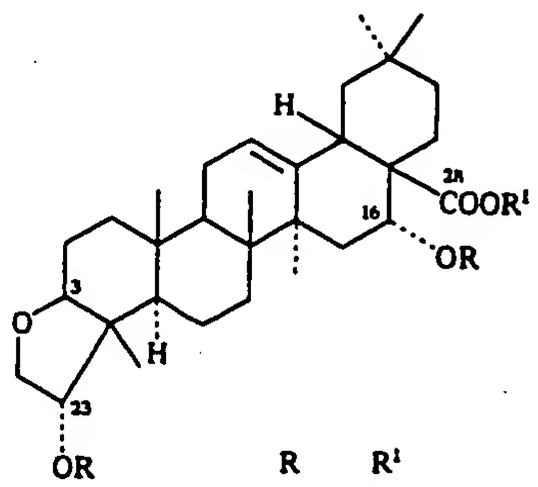
Reduction of **4** with sodium borohydride followed by methylation of the product by the Hakomori method [10] afforded compound **5**. Compound **5** was again reduced and methylated to yield compound **6**, which was methanolysed to give an aglycone (**7**) and three methylated sugars. An acetate (**8**) of **7** showed in the ^1H NMR spectrum the signals of one acetoxyl and three methoxyl groups, together with triplet-like signals at δ4.92 ascribable to the proton at C-3 bearing the acetoxyl group.



	R	R^1	R^2
9	Me	CH_2OMe	CH_2OMe
4	H	COOH	CHO
5	Me	COOMe	CH_2OMe
6	Me	CH_2OMe	CH_2OMe



	R	R^1	R^2
3	H	CHO	H
7	H	CH_2OMe	Me
8	Ac	CH_2OMe	Me



	R	R^1
10	H	Me
11	H	A
12	H	H
13	Me	B
14	Me	H
16	H	C
17	Me	D

Therefore 7 and 8 were regarded as the 16,23-di-*O*-methyl ether of 16α-hydroxyhederagenin methyl ester and 3-*O*-acetate, respectively. The methylated sugars were identified as methyl pyranosides of 2,3,4-tri-*O*-methyl-xylose (S-1), 2,3,4,6-tetra-*O*-methyl-galactose (S-2) and 2,4-di-*O*-methyl-glucose (S-3). Therefore, compound 4 has a branched trisaccharide, xylopyranosyl-[galactopyranosyl]-glucuronopyranose, combined with the 3-hydroxyl group of 3, and the Xyl and Gal units are attached to the 2- and 3-, or 3- and 2-hydroxyl groups of Glr, respectively.

When compound 6 was treated with dilute hydrochloric acid in methanol, a major product was obtained. Methylation of the product afforded compound 9, which gave on methanolysis compound 7, S-2 and methyl 3,4,6-tri-*O*-methyl-glucopyranose (S-4). This indicated compound 9 to be the permethylate of the galactosyl-(1 → 2)-glucoside of 16α-hydroxyhederagenin. Therefore, if Xyl, Gal and Glr are assumed to be the most commonly found series, the oligosaccharide moiety of 4 is D-galactopyranosyl-(1 → 2)-[D-xylopyranosyl-(1 → 3)]-D-glucuronopyranose. The ^{1}H NMR spectrum of 5 showed three anomeric proton signals of sugar units as doublets with large J values (7, 7 and 8 Hz) indicating their β-linkage [12].

Consequently, compound 4 is quillaic acid 3-*O*-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-D-glucuronopyranoside, and therefore compounds 1 and 2 are thought to be 28-*O*-glycosides of 4.

In the course of experiments concerning the structure elucidation of 4, we found and reported [13] that the sugar–aglycone linkage of 4 was cleaved to give the aglycone 10 (methyl 3β,16α,23α-trihydroxy-3-*O*,23-methylenolean-12-en-28-oate) and the corresponding oligosaccharide residue by only treatment of 4 with diazomethane–ether in methanol. If this procedure (diazomethane degradation) is applied to compounds 1 and 2, the 28-*O*-glycoside of 12 must be obtained.

Treatment of compound 1 with diazomethane–ether in methanol afforded a less polar compound (11). Compound 11 revealed an ester carbonyl absorption (1735 cm^{-1}) in the IR spectrum, the signals of four anomeric carbons in the ^{13}C NMR spectrum and a molecular ion peak at m/z 1079 $[M+Na]^+$ in the FAB mass spectrum, and gave on acid hydrolysis Fuc, Rham, Xyl, Api and an aglycone (12), which was converted to 10 by methylation with diazomethane. These data indicated that 11 is the 28-*O*-tetraglycoside of 12 obtained by cleavage of the 3-*O*-glycosidic linkage in 1, and that the sugar moiety of 11 consisted of 1 mol each of Fuc, Rham, Xyl and Api.

Methanolysis of the permethylate (13) of 11 gave 14 (the 16,23-di-*O*-methyl ether of 12), methyl 2,3,5-tri-*O*-methyl-apiofuranoside (S-5) and methyl pyranosides of 2,4-di-*O*-methyl-xylose (S-6), 2,3-di-*O*-methyl-rhamnose (S-7) and 3,4-di-*O*-methyl-fucose (S-8). This indicated that the sugar moiety of 11 is linear in structure and that apiofuranose is located at the terminal. Compound 11 was treated with 2% hydrochloric acid in methanol to yield 15 together with 12 and methyl apiofuranoside. Compound 15 showed a molecular ion peak at m/z 479 $[M+Na]^+$ in the FAB mass spectrum and revealed one methoxyl and three anomeric carbon signals in the ^{13}C NMR spectrum, and afforded Xyl, Rham and Fuc on acid hydrolysis. These data indicated that 15 was a methyl glycoside of a trisaccharide consisting of Xyl, Rham and Fuc. The detailed assignment of the ^{13}C NMR spectrum of 15 was made by taking the glycosylation shift [14, 15] into account and by comparison with the spectra of methyl fucopyranoside and gleditsia saponin [16] as shown in Table 1. The result of methanolysis of 13 indicated that if Xyl, Fuc and Rham are assumed to be the most commonly found D, D and L-series, compound 15 is the methyl glycoside of β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-D-fucopyranose. The above facts suggested that the sugar moiety of 11 is D-apiofuranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-D-fucopyranose since apiose in glycosides is also usually found as the D-series.

The configuration of the D-fucopyranose unit was regarded to be β by the J values of its anomeric proton signal (doublet, J = 8 Hz) [12] in the ^{1}H NMR spectrum of 13. The D-apiofuranose unit was considered to have the β-configuration by comparison of its anomeric carbon signal (δ 111.1) in the ^{13}C NMR spectrum of 11 with those of the methyl β- and α-D-apiofuranosides (β-anomer: δ 111.3; α-anomer: δ 104.4). Therefore, it follows that compound 11 is the 28-*O*-β-D-apiofuranosyl-(1 → 3)-β-D-

Table 1. ^{13}C NMR spectral data (C_5D_5N) of compounds 15, 18 and reference compounds

C	15	18	Reference compounds*
			[F]
1′	100.5	100.4	101.6
2′	78.4	77.9†	70.0
3′	69.9	69.9	71.6
4′	73.3	73.2	73.2
5′	66.6	66.6	66.9
6′	17.0	17.0	17.1
OMe	54.9	54.9	55.1
			[R]
1″	104.2	104.0	101.2
2″	71.6	71.1	71.9
3″	72.7	77.7†	72.6
4″	84.5	82.7	83.9
5″	68.1	68.1	68.4
6″	18.4	18.7	18.7
			[X]
1‴	107.0	105.1‡	106.9
2‴	76.0	75.4§	76.0
3‴	78.5	78.7	78.6
4‴	70.9	71.1	71.0
5‴	67.4	67.0	67.4
			[G]
1⁗		104.7‡	105.5
2⁗		75.2§	74.9
3⁗		78.7	78.3
4⁗		71.1	71.6
5⁗		78.7	78.3
6⁗		62.2	62.7

*[F]: Methyl α-D-fucopyranoside; [R] and [X]: rhamnose and xylose parts in β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl moiety in gleditsia saponin [16]; [G]: methyl β-D-glucopyranoside.

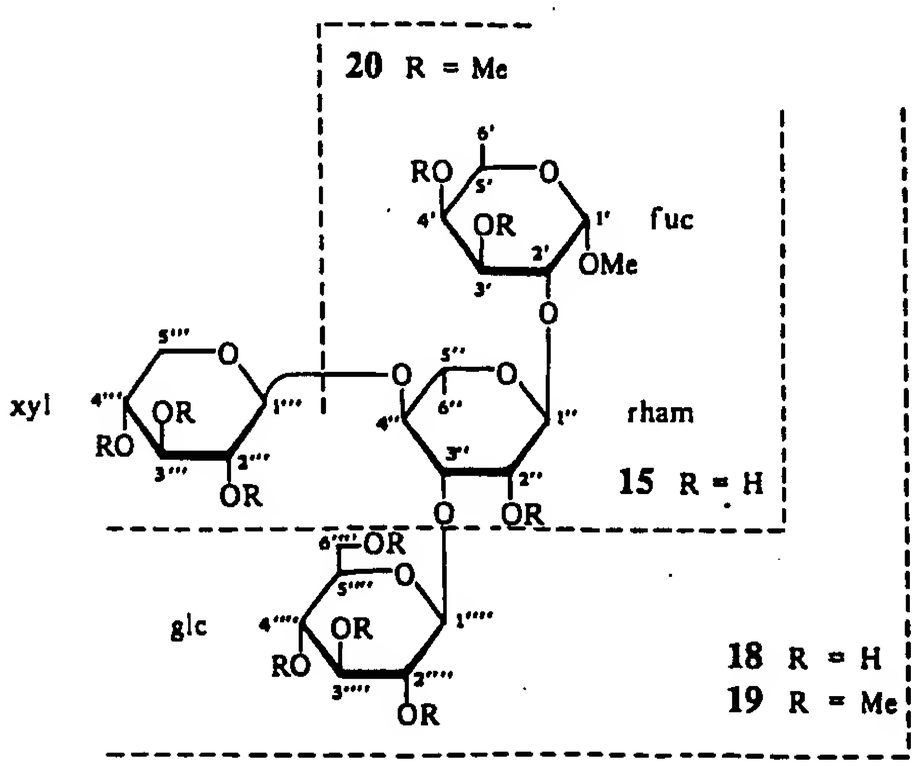
†,‡,§Assignments may be reversed in each vertical column.

xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside of **12**.

Consequently, due to the structures of compounds **4** and **11**, compound **1** is characterized as 3-*O*-β-D-galactopyranoysl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl quillaic acid 28-*O*-β-D-apiofuranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside.

The structure of the 28-*O*-oligosaccharide moiety in compound **2** was also determined by using the diazomethane degradation method as follows. On treatment with diazomethane, compound **2** afforded a less polar compound (**16**) which was presumed to be the 28-*O*-pentaglycoside of **12**. On acid hydrolysis **16** gave **12**, Fuc, Rham, Xyl, Api and Glc, and showed the molecular ion peak at *m/z* 1241 $[M+Na]^+$ in the FAB mass spectrum, five anomeric carbon signals in the ^{13}CNMR spectrum and an ester carbonyl absorption (1735 cm^{-1}) in the IR spectrum. These data indicated **16** is the 28-*O*-glycoside of **12** consisting of 1 mol each of **12**, Fuc, Rham, Xyl, Api and Glc. Methanolysis of the permethylate (**17**) of **16** afforded **14** and five methylated sugars, S-5, S-6, S-8 and the methyl pyranosides of 2-*O*-methyl-rhamnose (S-9) and 2,3,4,6-tetra-*O*-methyl-glucose (S-10). These facts and the co-existence of **1** and **2** in the same plant material suggested that the sugar moiety of **16** is a pentasaccharide in which a glucopyranose is located on the 3-hydroxyl group of the rhamnopyranose unit in the sugar moiety of **11**.

When compound **16** was treated with 2% hydrochloric acid in methanol in the same manner as **11**, compound **18** was obtained together with **12** and methyl apiofuranoside. Since **18** gave on acid hydrolysis Xyl, Rham, Fuc and Glc and showed the signals of one methoxyl and four anomeric carbons in the ^{13}CNMR spectrum and the molecular ion peak at *m/z* 619 $[M+H]^+$ in the FAB mass spectrum, compound **18** was regarded as the methyl glycoside of tetraose derived from the sugar moiety of **16**. The ^{13}CNMR signals of **18** were assigned as shown in Table 1 by taking the glycosylation shift into account and by comparison with the spectra of **15** and methyl glucopyranoside. The permethylate (**19**) of **18** was methanolysed to give S-8, S-9, S-10 and S-1. Therefore, if Xyl, Glc, Fuc and Rham are assumed to be the commonly found D, D, D and L-series, compound **18** must be methyl β-D-xylopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-D-fucopyranoside. The site of linkage of the Glc and Xyl units to the 3- and 4-hydroxyl groups of the rhamnose unit in **18** was confirmed as follows. Compound **19** was hydrolysed with 5% hydrochloric acid in methanol and the major product was methylated to give a compound (**20**) which showed no hydroxyl absorption in the IR spectrum and three anomeric proton signals in the ^1HNMR spectrum. Methanolysis of **20** afforded three methylated sugars, S-8, S-10 and methyl 2,4-di-*O*-methyl-rhamnopyranoside (S-11), which indicated that **20** is the permethylate of glucosyl-(1 → 3)-rhamnosyl-(1 → 2)-fucose.



Accordingly, compound **16** is the 28-*O*-apiofuranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-D-fucopyranoside of **12**. The configurations of both D-fucopyranose and D-apiofuranose were suggested to be β by the anomeric proton signal of fucopyranose (doublet, J = 8 Hz) [12] in the ^1HNMR spectrum of **17** and the anomeric carbon signals of apiofuranose (δ 111.2) in the ^{13}CNMR spectrum of **16**.

Based on the structure of **16**, compound **2** was regarded to be 3-*O*-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl quillaic acid 28-*O*-β-D-apiofuranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside.

To our knowledge, two quillaic acid 3,28-*O*-bisglycosides have been reported [9, 17], but the two desacylsaponins, **1** and **2**, reported here are different in their sugar moieties from those described so far. Diazomethane degradation was a useful method for structure elucidation of the triterpene 3,28-*O*-bisglycoside as described in this paper.

EXPERIMENTAL

All mps are uncorr. Optical rotations were recorded at 18–28° using a 1 dm cell. ^1HNMR spectra were taken at 100 MHz in $CDCl_3$ soln unless otherwise specified, using TMS as internal standard. ^{13}CNMR spectra were recorded at 25 MHz in C_5D_5N (TMS as internal standard) unless otherwise noted, employing the FT mode. The EI- and FABMS were measured on a double focusing mass spectrometer. The former were taken with an accelerating potential of 3–6.5 kV and an ionizing potential of 30–75 eV, and the latter at 1.5–3 kV for the ion source and 6 kV for an Ar beam source; the spectra were obtained from glycerol solns unless otherwise specified. FDMS were taken at 2–3 kV for the field anode and at −5 kV for the slotted cathode plate, at an ion source pressure of *ca* 10^{-7} Torr and an emitter heating current of 18–24 mA. Conditions of GLC (FID mode): (a) glass column (1.2 m × 3 mm) packed with 10% 1,4-butanediol succinate on Shimalite W (60–80 mesh), column temp. 155°; (b) glass column (1.2 m × 3 mm) packed with 1% neopentyl glycol succinate polyester on Chromosorb W(AW)-DMCS (60–80 mesh), column temp. 130°. Solvent systems of TLC [silica gel, C-8 (reversed phase) and Avicel]: (a) $CHCl_3$–MeOH–HOAc–H_2O (15:9:1:2); (b) EtOAc–MeOH–H_2O (8:1:0.1); (c) $CHCl_3$–MeOH (10:1); (d) C_6H_6–Me_2CO (2:1); (e) C_6H_6–Me_2CO (4:1); (f) *n*-hexane–EtOAc (2:1); (g) 60% MeOH; (h) upper layer of *n*-BuOH–pyridine–H_2O (6:2:3) + pyridine (1); (i) upper layer of *n*-BuOH–HOAc–H_2O (4:1:5).

Isolation of quillajasaponin. Commercial quillaja bark (2.5 kg) (a voucher specimen of the crude drug is on file at the Faculty of Pharmaceutical Sciences, Kyushu University) was extracted with MeOH and the MeOH was evaporated *in vacuo* to leave the MeOH extract (420 g). The latter was partitioned between EtOAc

and H_2O, and the H_2O layer was passed through an Amberlite XAD-2 column and eluted with H_2O and MeOH. Crude glycosides (85 g) obtained by evaporation of the MeOH eluate were treated with Sephadex LH-20 CC (eluate MeOH) to give two fractions, fraction 1 (39 g) (quillajasaponin) and 2 (42 g). Fraction 1 showing 7 spots (giving a dark-green colour after spraying with 5% H_2SO_4–MeOH followed by heating) on TLC [silica gel (a)] was refluxed with 3.5% HCl in 50% EtOH for 3 hr and the reaction mixture revealed a spot of quillaic acid (R_f 0.40) on TLC [silica gel (c)] (identified with an authentic sample). Fraction 2 showed two major spots (yellow colour by 5% H_2SO_4–MeOH and heating) on TLC [silica gel (a)] and further investigation was not carried out.

Alkaline hydrolysis of quillajasaponin. Quillajasaponin (18 g) was boiled with 6% $NaHCO_3$ in 50% MeOH (300 ml) for 1 hr, and the reaction mixture was neutralized with Dowex 50W-X8 and filtered. The filtrate showing spots of less polar and polar compounds on TLC [silica gel (a)] was evaporated *in vacuo* and the residue was chromatographed on Sephadex LH-20 (MeOH) to give two fractions, fractions 1 (5.0 g) (polar compounds, desacylsaponins) and 2 (2.2 g) (less polar compounds, eliminated acyl groups). Fraction 1 showing two major spots (R_f 0.11, 0.07) on TLC [silica gel (a)] was chromatographed on silica gel (eluant $CHCl_3$–MeOH–HOAc–H_2O, 15:9:1:2) to give two fractions, fractions 1′ (R_f 0.11) and 2′ (R_f 0.07). Each fraction showed a major and a few minor spots on TLC [C-8(g)] and each was chromatographed on C-8 (eluant 50% MeOH) to give a major component, DS-1 (1, 1.4 g) and DS-2 (2, 2.0 g) [R_f 0.11 (1) and 0.07 (2), silica gel (a); R_f 0.29 (1) and 0.35 (2), C-8 (g)].

DS-1 (1). White powder (from *n*-BuOH–H_2O–MeOH), mp 255–258° (decomp.), $[\alpha]_D$ −19.6° (H_2O; *c* 0.67). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (carbonyl), 1610 (COO^-). FABMS (using KI as additive) *m/z*: 1589 $[M(C_{69}H_{108}O_{36})+2K-H]^+ = [(C_{68}H_{107}O_{34}\cdot COOK)+K]^+$. ^{13}C NMR: δ94.8, 101.0, 103.1, 103.9, 104.5, 106.9, 111.0 (each *d*, anomeric C × 7), 176.0 (*s*, C-28), 209.8 (*d*, C-23). Compound 1 (40 mg) was refluxed with 2 N H_2SO_4 in 50% EtOH for 8 hr and then diluted with H_2O, and the ppt. was collected by filtration. The ppt. (crude aglycone) (10 mg) was chromatographed on silica gel ($CHCl_3$–MeOH, 30:1 → 15:1) to provide 3 as colourless needles (from MeOH–H_2O), mp 256–260°, $[\alpha]_D$ +66.7° (MeOH; *c* 1.02). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1730, 1715 (carbonyl). EIMS *m/z*: 486$[M(C_{30}H_{46}O_5)]^+$, 264, 246. Compound 3 was identified with an authentic sample of quillaic acid, by mmp, TLC, IR and ^{13}C NMR. The H_2O layer was neutralized with $Ba(OH)_2$ soln, filtered and the filtrate was concentrated. The residue was examined by TLC [Avicel (h) and (i)], and Glr, Gal, Xyl, Fuc, Rham and Api were detected.

DS-2 (2). White powder (from *n*-BuOH–H_2O–MeOH), mp 258–261° (decomp.), $[\alpha]_D$ −24.7° (H_2O; *c* 1.00). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (carbonyl), 1610 (COO^-). FABMS (using KI as additive) *m/z*: 1751 $[M(C_{75}H_{118}O_{41})+2K-H]^+ = [(C_{74}H_{117}O_{39}\cdot COOK)+K]^+$. ^{13}C NMR: δ95.0, 101.9, 103.1, 103.6, 104.3, 104.5, 104.9, 110.8 (each *d*, anomeric C × 8), 176.3 (*s*, C-28), 210.2 (*d*, C-23). On hydrolysis with acid under the same conditions as those for 1, compound 2 gave 3 and a sugar mixture. The sugar mixture was found to consist of Glr, Gal, Xyl, Fuc, Rham, Api and Glc [TLC, Avicel (h) and (i)].

Akaline hydrolysis of 1 *and* 2 *providing* 4. Compound 1 (200 mg) was refluxed with 2% KOH in 50% EtOH (10 ml) for 7 hr and the reaction mixture was neutralized with dilute HCl and concentrated *in vacuo*. The residue showing a spot (R_f 0.40) on TLC [silica gel (a)] was passed through a Sephadex G-15 column (H_2O) and a silica gel column ($CHCl_3$–MeOH–HOAc–H_2O, 15:9:1:2) to give a white powder (4) (from *n*-BuOH–H_2O–MeOH) (80 mg), mp > 300° (decomp.), $[\alpha]_D$ +18.6° (H_2O; *c* 0.73). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1710 (carbonyl), 1610 (COO^-). FABMS *m/z*: 995 $[M(C_{47}H_{72}O_{20})+K]^+$. ^{13}C NMR ($C_5D_5N+D_2O$): δ24.6 (*t*, C-2), 54.9 (*s*, C-4), 84.2 (*d*, C-3), 102.7, 102.9, 103.6 (each *d*, anomeric C × 3), 180.1 (*s*, C-28), 210.1 (*d*, C-23). Compound 4 was hydrolysed with acid in the same manner as for 1 to provide 3 and sugars. The sugars were identified as Glr, Gal and Xyl [TLC, Avicel (h) and (i)]. Compound 2 (500 mg) was hydrolysed with alkali and worked up in the same manner as that for 1 to provide a white powder (200 mg) which was identical with 4 (mmp, TLC, IR and ^{13}C NMR).

Preparation of 6 *from* 4. $NaBH_4$ (1.6 g) was added to a soln of 4 (250 mg) in 50% MeOH (25 ml) under ice-cooling and the reaction mixture was stirred for 2 hr at room temp. Me_2CO (2 ml) was added to the mixture and concentrated *in vacuo*, and the residue was passed through a Diaion HP20AG column (eluant H_2O → MeOH). The MeOH eluate showing a spot (R_f 0.36) on TLC [silica gel (a)] was evaporated *in vacuo* to give a white powder (230 mg), mp 275–277° (decomp), $[\alpha]_D$ +3.8° (H_2O; *c* 0.9). ^{13}C NMR: δ64.2 (*t*, C-23), 175.0 (*s*, COOH of Glr), 182.4 (*s*, C-28), no CHO. The product (200 mg) was treated with NaH (500 mg) and MeI (5 ml) in DMSO (20 ml) (Hakomori method) and the reaction mixture was diluted with H_2O, extracted with Et_2O and the Et_2O layer was washed, dried and evaporated. The residue was chromatographed on silica gel (C_6H_6–Me_2CO, 7:1) to give a major product as a white powder (5) (150 mg). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1760 (Glr-COOMe) [11], 1720 (28-COOMe) [18], no OH. ^1H NMR: δ4.35 (1H, *d*, *J* = 7 Hz, anomeric H), 4.87 (1H, *d*, *J* = 7 Hz, anomeric H), 4.96 (1H, *d*, *J* = 8 Hz, anomeric H). FDMS *m/z*: 1127 $[M(C_{59}H_{98}O_{20})+H]^+$. Compound 5 (100 mg) in MeOH (10 ml) was reduced with $NaBH_4$ (1.2 g) as for 4. Me_2CO (2 ml) and H_2O (2 ml) were added to the mixture and evaporated *in vacuo*, and the residue was diluted with excess H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was washed, dried and concentrated, and the residue showing a spot (R_f 0.53) (R_f of 5, 0.77) on TLC [silica gel (d)] was methylated by the Hakomori method. The crude methylated product was chromatographed on silica gel (C_6H_6–Me_2CO, 7:1) to give 6 as a white powder (23 mg). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1720 (28-COOMe), no OH. ^1H NMR: δ3.30 (6H, *s*, OMe × 2), 3.35, 3.37, 3.46 (each *s*, 3H, OMe × 3), 3.50 (9H, *s*, OMe × 3), 3.53, 3.55 (each *s*, 3H, OMe × 2), 3.59 (6H, *s*, OMe × 2), 4.24 (1H, *d*, *J* = 7 Hz, anomeric H), 4.92 (1H, *d*, *J* = 7 Hz, anomeric H), 5.02 (1H, *d*, *J* = 8 Hz, anomeric H).

Methanolysis of 6. Compound 6 (35 mg) was boiled with 10% HCl in MeOH (4 ml) for 2 hr, the mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated and the residue (methanolysate) was examined by TLC [silica gel (f) and (d)] and GLC [conditions (a) and (b)]; one aglycone (7) and three methylated sugars were detected. The sugars were identified as S-1, S-2 and S-3 by direct comparison with authentic samples. The methanolysate was chromatographed on silica gel (C_6H_6–Me_2CO, 80:1 → Me_2CO) to give 7 (10 mg). Compound 7 was acetylated with Ac_2O–pyridine as usual to give an acetate (8) (5 mg) as colourless needles (from MeOH–Me_2CO), mp 207–210°. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730 (ester), no OH. EIMS *m/z*: 572 $[M(C_{35}H_{56}O_6)]^+$, 292. ^1H NMR: δ2.04 (3H, *s*, OAc), 2.93 and 3.10 (1H each, *d*, *J* = 10 Hz, H_2-23), 3.25, 3.29, 3.59 (each *s*, 3H, OMe × 3), 3.91 (1H, *br s*, H-16), 4.92 (1H, *t*-like, *J* = 8 Hz, H-3).

Preparation and methanolysis of 9. Compound 6 was heated with 2% HCl–MeOH for 8 hr at 60° and worked up as before. The mixture showing two major spots [R_f 0.28(6), 0.14] on TLC (silica gel; C_6H_6–Me_2CO, 5:1) was chromatographed on silica gel (*n*-hexane–Me_2CO, 5:1) to give a compound (R_f 0.14) which was methylated by the Hakomori method and worked up as before to yield 9 as a white powder. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1720 (ester), no OH. ^1H NMR: δ3.28 (6H, *s*, OMe × 2), 3.32, 3.35, 3.39 (each *s*, 3H, OMe × 3), 3.51 (9H, *s*, OMe × 3), 3.60, 3.65 (each *s*, 3H, OMe × 2),

4.24 (1H, *d*, $J = 8$ Hz, anomeric H), 4.62 (1H, *d*, $J = 7$ Hz, anomeric H). Compound 9 was subjected to methanolysis as for 6, and 7: S-2 and S-4 were obtained [TLC silica gel (f), GLC (a)].

Diazomethane degradation of 1 *providing* 11. A soln of CH_2N_2 in Et_2O (150 ml) was poured into a soln of 1 (1.1 g) in MeOH (300 ml) under cooling with ice. The mixture was left to stand for 1 hr at room temp., excess CH_2N_2 was decomposed with HOAc and the solvents were removed by distillation. The crude reaction mixture revealing two major spots (R_f 0.51 and 0.27) in TLC [silica gel (a)] was chromatographed on silica gel ($CHCl_3$–MeOH–H_2O, 8:3:0.3) to give a substance (white powder, R_f 0.27) (a mixture of the related compounds of the methyl ester of 1 and trisaccharides derived from the 3-*O*-sugar residue in 1 [13]) and 11 (white powder, 164 mg, R_f 0.51), mp 198–201° (decomp.), $[\alpha]_D$ −30.4° (MeOH; *c* 1.65). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1735 (ester). FABMS *m/z*: 1079 $[M(C_{53}H_{84}O_{21}) + Na]^+$. ^{13}C NMR: δ94.8, 101.2, 106.9, 111.1 (each *d*, anomeric C × 4), 176.1 (*s*, C-28). Compound 11 was boiled with 2 N H_2SO_4 for 2 hr and the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ layer showing a spot (R_f 0.21) on TLC [silica gel (e)] was washed, dried and evaporated to give 12, which was methylated with CH_2N_2–Et_2O in MeOH affording a compound corresponding with an authentic sample of 10 [TLC (e), IR, ^1H NMR and ^{13}C NMR]. The aq. layer was treated as for 1 and Fuc, Rham, Xyl and Api were detected [TLC, Avicel (h) and (i)].

Synthesis and methanolysis of the permethylate (13) *of* 11. Compound 11 (40 mg) was methylated by the Hakomori method as before, and the reaction mixture was diluted with H_2O, extracted with $CHCl_3$ and the $CHCl_3$ layer was washed, dried and evaporated. The residue was passed through silica gel (*n*-hexane–Me_2CO, 2:1) and a Sephadex LH-20 (MeOH) column to give 13 as a white powder (16 mg). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1750 (ester), no OH. FDMS *m/z*: 1210 $[M(C_{64}H_{106}O_{21})]^+$. ^1H NMR: δ4.63 (1H, *d*, $J = 8$ Hz, anomeric H of Xyl), 5.24 (1H, *d*, $J = 1$ Hz, anomeric H of Rham), 5.37 (1H, *d*, $J = 8$ Hz, anomeric H of ester glycosidic fucose [19]), 5.46 (1H, *d*, $J = 2$ Hz, anomeric H of Api [20]) (anomeric H signals were assigned by comparison with those observed in the ^1H NMR spectrum of 17, and with the reported δ and *J* values [19, 20]). Compound 13 was methanolysed and worked up in the same manner as that for 6. The methanolysate was examined by TLC [silica gel (d)] and GLC [condition (a)], and S-5, S-6, S-7 and S-8 were detected. Silica gel CC of the methanolysate (*n*-hexane–Me_2CO, 6:1) afforded an aglycone (14). EIMS *m/z*: 528 $[M(C_{33}H_{52}O_5)]^+$, 278.

Partial methanolysis of 11 *yielding* 15. Compound 11 (300 mg) in 2% HCl–MeOH (20 ml) was left to stand for 2.5 hr at 40°, and the reaction mixture was treated with Ag_2CO_3, filtered and evaporated. The residue giving three spots [R_f 0.84(12), 0.61 (methyl β-D-apiofuranoside) and 0.31] on TLC [silica gel (a)] was chromatographed on silica gel ($CHCl_3$–MeOH–HOAc–H_2O, 15:8:1:1) and Sephadex LH-20 (MeOH) to yield 15 (R_f 0.31) (31 mg) as a hygroscopic powder, $[\alpha]_D$ +11.8° (MeOH; *c* 1.00). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH). FAB MS *m/z*: 479 $[M(C_{18}H_{32}O_{13}) + Na]^+$. ^{13}C NMR: see Table 1. Compound 15 was boiled with 2 N H_2SO_4 for 2 hr and treated as usual. The hydrolysate was examined by TLC [Avicel (h) and (i)], and Xyl, Rham and Fuc were detected.

Diazomethane degradation of 2 *affording* 16. Compound 2 (2.0 g) in MeOH (150 ml) was treated with CH_2N_2–Et_2O (130 ml) and worked up as for 1. The crude reaction mixture showing three spots (R_f 0.42, 0.27 and 0.21) on TLC [silica gel (a)] was chromatographed on silica gel ($CHCl_3$–MeOH–H_2O, 6:4:0.3) to give substances of R_f 0.27 (3-*O*-trisaccharide residues in 2), R_f 0.21 (related compounds of the methyl ester of 2) and R_f 0.42 (16) (a white powder, 508 mg), mp 211–214° (decomp.), $[\alpha]_D$ −40.4° (MeOH; *c* 3.23). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1735 (ester). FAB MS *m/z*: 1241 $[M(C_{59}H_{94}O_{26}) + Na]^+$. ^{13}C NMR: δ95.1, 102.0, 104.8, 105.2, 111.2 (each *d*, anomeric C × 5), 175.9 (*s*, C-28). On hydrolysis with acid under the same conditions as for 11, 16 gave 12 and a sugar mixture. The sugar mixture was found to consist of Fuc, Rham, Xyl, Api and Glc [TLC, Avicel (h) and (i)].

Preparation and methanolysis of 17. Compound 16 (300 mg) was methylated by the Hakomori method and worked up in the same manner as for 11 to provide a permethylate (17) (88 mg) as a white powder. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1755 (ester), no OH. FDMS *m/z*: 1414 $[M(C_{73}H_{122}O_{26})]^+$. ^1H NMR: δ4.68 (1H, *d*, $J = 7$ Hz, anomeric H of xylose), 4.82 (1H, *d*, $J = 7$ Hz, anomeric H of Glc), 5.05 (1H, *d*, $J = 2$ Hz, anomeric H of Rham), 5.39 (1H, *d*, $J = 8$ Hz, anomeric H of Fuc), 5.41 (1H, *d*, $J = 2$ Hz, anomeric H of Api) (anomeric H signals were assigned by comparison with the ^1H NMR spectrum of 13). Compound 17 was methanolysed and the methanolysate was examined as for 13. Compound 14, S-5, S-6, S-8, S-9 and S-10 were detected [TLC silica gel (d), GLC (a)].

Partial methanolysis of 16 *yielding* 18. Compound 16 (450 mg) was treated with 2% HCl–MeOH for 1.5 hr and worked up as for 11. The residue showing the spots of 12, methyl apiofuranoside and 18 (R_f 0.22) on TLC [silica gel (a)] was chromatographed on silica gel (EtOAc–MeOH–H_2O, 6:4:0.4) and Sephadex LH-20 (MeOH) to give 18 (145 mg) as a white powder, mp 178–182°, $[\alpha]_D$ −3.2° (MeOH; *c* 3.73). IR ν_{max}^{KBr} cm^{-1}: 3380 (OH). FABMS *m/z*: 619 $[M(C_{24}H_{42}O_{18}) + H]^+$. ^{13}C NMR: see Table 1. Compound 18 was hydrolysed with acid and worked up as for 15; Xyl, Glc, Rham and Fuc were obtained [TLC Avicel (h) and (i)].

Synthesis of 19 *and its methanolysis.* In the same manner as that for 11, compound 19 (63 mg) was prepared from 18 (120 mg) by the Hakomori method. IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH. EIMS *m/z*: 758 $[M(C_{34}H_{62}O_{18})]^+$. Compound 19 was methanolysed as for 13 to give S-8, S-9, S-10 and S-1 [TLC silica gel (d), GLC (a)].

Preparation of 20 *and its methanolysis.* Compound 19 (50 mg) was methanolysed with 5% HCl–MeOH (3 ml) for 3 hr at 50° and worked up as before, and the residue (partial methanolysate) was chromatographed on silica gel (*n*-hexane–Me_2CO, 3:1) to give a major product (R_f 0.09) [TLC silica gel (*n*-hexane–Me_2CO, 3:1)] (R_f of 19, 0.18). The major product was methylated by the Hakomori method and treated as usual to yield 20 (8 mg) as a colourless syrup. IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH. ^1H NMR: δ3.33, 3.34, 3.47, 3.53, 3.57, 3.60, 3.62 (each *s*, 3H, OMe × 7), 3.52 (6H, *s*, OMe × 2), 4.45 (1H, *d*, $J = 7$ Hz, anomeric H), 4.77 (1H, *d*, $J = 4$ Hz, anomeric H), 4.95 (1H, *d*, $J = 2$ Hz, anomeric H). Methanolysis of 20 as before afforded S-8, S-10 and S-11 [TLC silica gel (d), GLC (a)].

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Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function

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QS-21, a purified Quillaja saponaria *saponin immunologic adjuvant, contains two functional groups that we hypothesized to be involved in the adjuvant mechanism of action through charge or Schiff base interaction with a cellular target. Derivatives, prepared by modification of these sites, were prepared and tested for their ability to augment the immunogenicity of the antigen ovalbumin (OVA) in C57BL/6 mice. QS-21 derivatives that were modified at the carboxyl group on an anionic sugar, glucuronic acid, retained adjuvant activity for antibody stimulation, inducing relative increases in antibody titers similar to those induced by QS-21, although the minimum adjuvant dose required for this stimulation was increased several fold relative to the dose of unmodified QS-21. One of these derivatives also retained significant activity for induction of OVA-specific cytotoxic T-lymphocytes. In contrast, QS-21 derivatives modified at an aldehyde on the triterpene did not show adjuvant activity for antibody stimulation or for induction of cytotoxic T-lymphocytes, suggesting that this functional group may be involved in the adjuvant mechanism.*

Keywords: QS-21; adjuvant; *Quillaja saponaria*; saponin; structure/function

Extracts of the bark of the South American tree *Quillaja saponaria* Molina contain a heterogeneous saponin fraction with potent adjuvant activity[1,2]. These saponins have been purified to near homogeneity by HPLC and characterized for adjuvant activity[3]. Several were shown to stimulate high antigen-specific antibody titers in mice[3,4]. One of these saponins, QS-21, was also shown to induce class I MHC-restricted cytotoxic T-lymphocyte (CTL) responses in mice when used with subunit antigens such as ovalbumin and recombinant HIV-1 envelope antigens[5,6] and to increase antibody titers to T-independent polysaccharide antigens[7]. QS-21 has also been tested as an adjuvant in a Phase I melanoma vaccine clinical trial and was noted to augment antigen-specific IgG titers[8], making it of particular interest as a vaccine adjuvant. However, relatively little is known of the minimum critical structure of QS-21 required for these adjuvant functions. This study addresses the relationship of QS-21 structure to its adjuvant function via analysis of two functional groups.

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QS-21 is a highly complex triterpene glycoside (*Figure 1*), with branched sugar chains at carbon 3 and carbon 28 on the triterpene quillaic acid[4]. A correlation between the presence of branched sugar chains at these positions and adjuvant activity of naturally occurring saponins was noted[9]. The glycoside on carbon 3 contains an anionic sugar residue, glucuronic acid, which imparts an overall negative charge to the QS-21 molecule at physiological pH. There is also an aldehyde on carbon 4 of the triterpene. In addition, QS-21 contains a fatty acid (3,5-dihydroxy-6-methyl-octanoic acid) linked through an ester bond to the 3-hydroxyl or 4-hydroxyl of fucose (N. Jacobsen, personal communication). An identical fatty acid is linked in ester bond to the 5-hydroxyl of the first fatty acid; the 5-hydroxyl of the second fatty acid is glycosylated with a single sugar (arabinose).

Relatively little is known of the minimum critical structure of QS-21 required for adjuvant function. There are several adjuvant active saponins that have been isolated from *Quillaja saponaria* Molina[3]. These include the saponins QS-7, QS-17, QS-18, and QS-21 which are the predominant saponins in the bark and which as an aggregate represent approximately half of the saponins present in *Quillaja saponaria* bark. Structural comparison suggests that the known adjuvant active saponins have the triterpene backbone (quillaic acid) and some

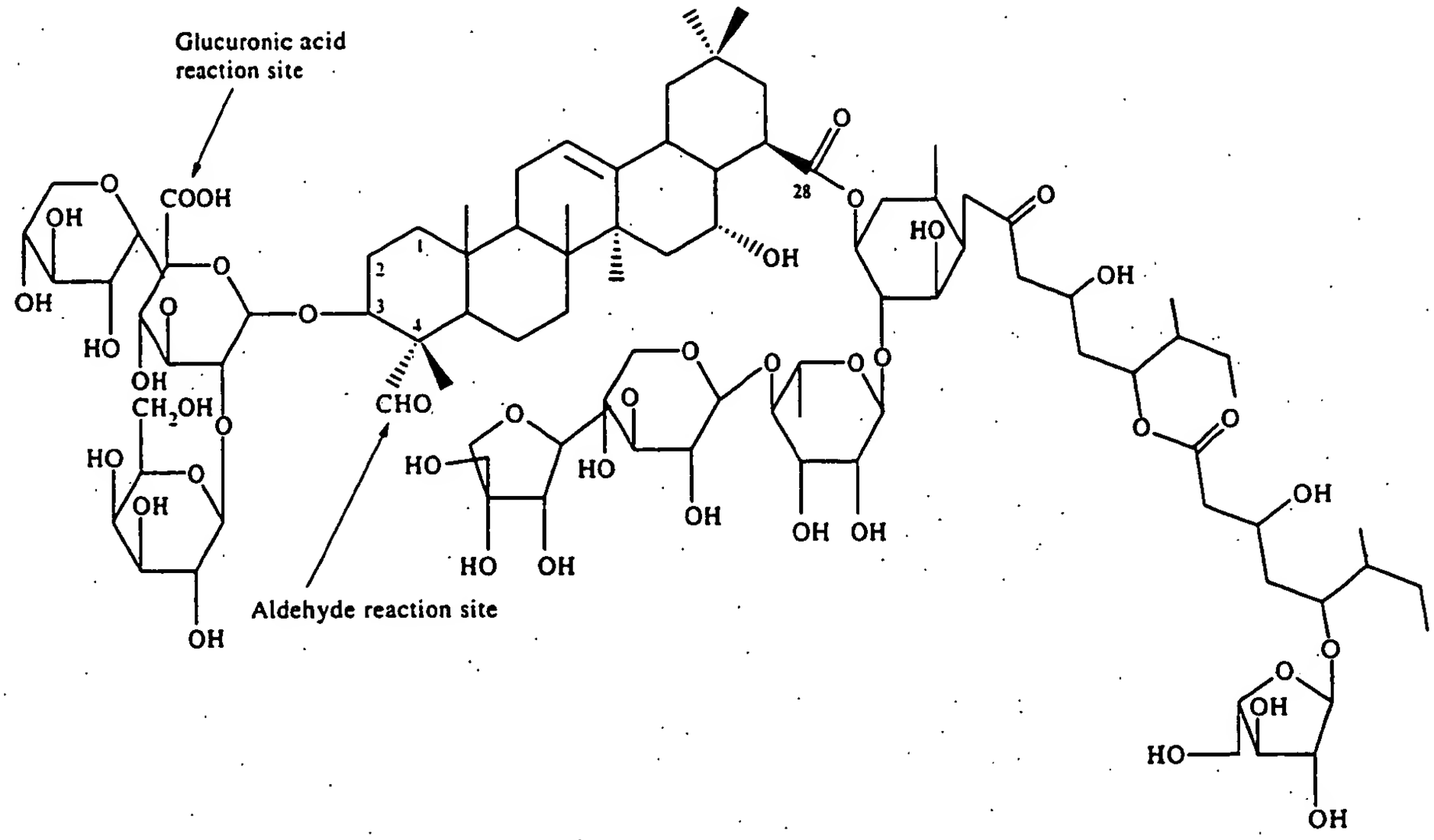

Figure 1 Structure of QS-21. The two functional groups selected for modification are shown

carbohydrate residues in common. Two structural features held in common by all adjuvant active saponins from *Quillaja saponaria* Molina are the 2,3 glucuronic acid[3,4,10] and the quillaic acid backbone, including the aldehyde at carbon 4[11]. We postulated that these functional groups were involved in the QS-21 adjuvant mechanism, the glucuronic acid through charge interaction and the aldehyde via Schiff base formation with a cellular target. Hence, these two functional groups (glucuronic acid carboxyl group and triterpene aldehyde) were modified by conjugation to small blocking groups; the resulting derivatives were tested as adjuvants in an effort to evaluate the importance of these functional groups or nearby regions to adjuvant function.

MATERIALS AND METHODS

Materials

QS-21 was purified from an aqueous extract of *Quillaja saponaria* bark by adsorption chromatography and reversed-phase HPLC[3]. N-hydroxysulfosuccinimide (S-NHS) and N,N′-dicyclohexylcarbodiimide (DCC) were purchased from Pierce Chemical Co. (Rockford, IL). Sodium cyanoborohydride, anhydrous dimethylformamide (DMF), anhydrous dimethylsulfoxide (DMSO), and 8-anilino-1-naphthalene-sulfonic acid (ANS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ovalbumin (OVA), Grade VI, was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of derivatives modified at glucuronic acid carboxyl

Conjugates (1:1 mol:mol) of QS-21 and the small molecules ethylamine, glycine, and ethylenediamine were prepared. In order to react the glucuronic acid carboxyl group of QS-21 with free amino groups on the molecules used as blocking agents, an active ester derivative of QS-21 was prepared using sulfo-NHS[12]. A twofold molar excess of S-NHS and a threefold molar excess of DCC were added to 50 mM QS-21 in anhydrous DMF; this reaction mixture was incubated with stirring overnight at room temperature to generate the S-NHS active ester derivative of QS-21 (QS-21/S-NHS ester). The reaction mixture was then chilled on ice and filtered to remove dicyclohexylurea. The active ester derivative of QS-21 was then precipitated by addition of ethylacetate (final ratio=6/1 ethylacetate/DMF, v/v), purified by repeated washes with ethylacetate, collected by centrifugation (15 min at 1000*g*), and dried by vacuum desiccation overnight.

In order to conjugate the small molecules glycine, ethylamine, and ethylenediamine through their free amino groups to the glucuronic acid carboxyl of QS-21, a modification of the method of Anjaneyulu and Staros[13] was employed. A 100-fold molar excess of glycine, ethylamine, or ethylenediamine (1.0 M in 0.1 M sodium phosphate, pH 7.0) was added to solid QS-21/S-NHS active ester. The reaction mixture was stirred at room temperature for 1 h. The resulting conjugates were purified on a Vydac C4 column (1.0 cm I.D. × 25 cm length, 5 μm particle size, 300 angstrom pore size) using a Waters 600E HPLC system and detection at 214 nm (LambdaMax Model 481 Variable Wavelength Detector). A linear water/acetonitrile gradient in 0.15% trifluoroacetic acid was used. Pooled fractions containing the predominant reaction product were lyophilized to dryness.

Preparation of derivatives modified at triterpene aldehyde

To conjugate glycine, ethylamine, and ethylenediamine through their free amino groups to the triterpene aldehyde on QS-21 (*Figure 1*), a 50-fold molar excess of these compounds in 0.1 M sodium phosphate, pH 6.0

was added to QS-21 (12 mM) in 0.1 M sodium phosphate (pH 6.0)/methanol (50/50, v/v) and incubated with stirring overnight at room temperature to induce Schiff base formation. These adducts were stabilized by the use of sodium cyanoborohydride as a selective reducing agent[14]. Sodium cyanoborohydride (from a 0.1 M stock solution in methanol) was added to a final ratio of 4/1 (mol/mol) over QS-21. The reaction mixture was stirred overnight. The predominant reaction product was purified by HPLC as described above.

Characterization of QS-21 derivatives

Derivatives were characterized for purity and retention time relative to QS-21 by reversed-phase HPLC on C18 (3 μm particle size, 120 angstrom pore size, 4.6 mm I.D. × 15.0 cm length (YMC Inc., Wilmington, N.C.)) using a linear gradient of 80% solvent A/20% solvent B to 40% solvent A/60% solvent B over 20 min at a 1 ml min^{-1} flow rate. Solvent A was 0.1% H_3PO_4 in water and solvent B was 0.1% H_3PO_4 in acetonitrile. Detection was by UV absorbance at 205 nm. Relative retention time was determined from the ratio of k'derivative/k'QS-21 where k'=(peak retention time −void retention time)/(void retention time). Molecular weights were determined by fast atom bombardment-mass spectrometry (M-Scan Corp., Westchester, PA) to confirm that these derivatives were 1:1 covalent conjugates. ^{1}H-NMR on samples in deuterated dimethylsulfoxide was carried out by Spectral Data Services (Champaign, IL). Modification of the triterpene aldehyde in compounds (5)–(7) was confirmed by elimination of the aldehyde proton resonance (singlet with chemical shift =9.47 in unmodified QS-21). The aldehyde proton resonance was present in compound (3) whereas a proton singlet at 8.1 ppm (assigned as the proton resonance on the amide bond nitrogen) appeared. Compounds (4) and (5) were not assayed by NMR.

Immunizations

C57BL/6 mice (female, 8–10 weeks of age) were used for all immunizations. Mice were immunized subcutaneously with 0.2 ml of 25 μg OVA and varying amounts of QS-21 derivatives with either two or three immunizations spaced 2–3 weeks apart. Sera were collected one week after the second immunization or two weeks after the third immunization for analysis by EIA. Splenic mononuclear cells for use as the source of effector cells in the CTL assay were collected two weeks after the last of three immunizations.

Immunological assays

The EIA was done using OVA-coated Immulon IV plates (Dynatech Laboratories, Chantilly, VA). Plates were coated with OVA by overcoating wells with 100 μl per well of 10 μg ml^{-1} OVA in PBS and incubation at 4°C overnight. Plates were emptied and were then incubated for 1 h at ambient temperature with 150 μl $well^{-1}$ of 10% normal goat serum (Gibco Laboratories, Grand Island, NY) in PBS. Plates were washed three times with 0.05% Tween-20 in water. Serial dilutions of sera in 10% normal goat serum in PBS (1/10 dilutions) were prepared and incubated on the plate for 1 h at room temperature. Plates were then washed three times with 0.05% Tween-20 in water. For measurement of total IgG, a total volume of 100 μl goat anti-mouse IgG-horseradish peroxidase conjugate (BioRad, Richmond, VA), diluted 1/ 12 500 in 10% normal goat serum in PBS, was incubated on the plate for 1 h at room temperature. For measurement of IgG1, $IgG2_b$, or $IgG2_a$, a volume of 100 μl goat-antimouse IgG1, $IgG2_b$, or $IgG2_a$ (conjugated to alkaline phosphatase, Southern Biotechnology, Birmingham, AL, diluted 1/250 in 10% normal goat serum/PBS) was added to each well and incubated at 4°C overnight. Plates were washed five times with 0.05% Tween-20 in water followed by one wash with water. Color development was with tetramethylbenzidine substrate for total IgG and with *p*-nitrophenylphosphate for IgG1, $IgG2_b$, and $IgG2_a$.

CTL activity was assayed using splenic mononuclear cells as the source of CTL. The precursor CTL in splenic mononuclear cell preparations were induced to mature *in vitro* by culture with mitomycin C-treated E.G7-OVA cells at a 20:1 (responder:stimulator) ratio or with denatured ovalbumin. For a negative antigen control, splenic mononuclear cells were cultured with medium (supplemented RPMI 1640). Culture in presence or absence of antigen was carried out using supplemented RPMI 1640 medium at 37°C in a 2 ml volume with 1×10^6 cells per ml in culture tubes. Cells were recovered after 144 h of culture and used in the CTL assay. The CTL activity was measured using both EL4 cells and E.G7-OVA (EL4 cells transfected with cDNA coding for OVA[15]) as targets. Cytotoxicity was measured after 4 h of incubation of CTL with 10^4 ^{51}Cr-labeled target cells per well, using effector/target (E/T) ratios of 25:1 to 3:1. The percent of specific ^{51}Cr release was calculated as 100 × (experimental release −spontaneous release)/(maximum release −spontaneous release) where maximum release was measured after lysis of target cells with 1% NP-40 and spontaneous release was measured after incubation of target cells with medium.

Hemolysis and critical micellar concentration assays

Hemolytic activity of QS-21 and derivatives were measured in an *in vitro* assay on sheep red blood cells. Five milliliters of sheep red blood cells in Alsever's solution (Biowhittaker, Walkersville, Maryland) were spun at 900*g* for 5 min. The pellet was resuspended in 5 ml PBS. This process was repeated twice. The final pellet was resuspended in 3 ml PBS. One hundred microliters of serial 1:2 dilutions of QS-21 or derivatives in PBS were added to individual wells of Falcon flexible round bottom 96 well assay plates (Becton Dickinson, Oxnard, CA). Twenty-five microliters of the washed red blood cell suspension were added to each well, mixed with the saponin solution, and incubated at room temperature for 30 min. The round bottom plate was then centrifuged at 1000*g* for 5 min. Fifty microliters of each of the supernatants were transferred to wells in a flat bottom microtiter plate for determination of the absorbance at 570 nm.

Critical micellar concentration (cmc) of QS-21 and derivatives was determined by a fluorescent dye binding assay as described previously[16,17]. The fluorescent probe ANS, 11 μM in phosphate-buffered saline, was mixed with different concentrations of QS-21 or derivative.

Immediately after mixing, fluorescence emission at 490 nm with excitation at 370 nm was determined. The fluorescence emission was plotted versus QS-21 or derivative concentration. Biphasic curves were obtained with low fluorescence emission below the cmc and significant increase in fluorescence emission above the cmc due to intercalation of ANS into the micelle. Best fit lines were determined for the biphasic curve; the cmc was defined as the QS-21 or derivative concentration corresponding to the intersection of the best fit lines.

RESULTS

Modification of QS-21

In order to evaluate the importance of the glucuronic acid and triterpene aldehyde to the adjuvant activity of QS-21, derivatives consisting of 1:1 conjugates of QS-21 linked to the small blocking groups glycine, ethylamine, and ethylenediamine at these functional groups were prepared. The size, charge, and hydrophobicity of these derivatives compared to QS-21 are summarized in *Table 1*. Although the size increase to QS-21 due to conjugation of these blocking groups was minimal, all of the conjugations were expected to sterically hinder or block any direct interactions at the modified sites. Some of these modifications also modified QS-21 charge, enabling an evaluation of whether the anionic carboxyl group on QS-21 was involved in a charge interaction as part of the adjuvant mechanism. In compound (3), the glucuronic acid was blocked with a neutral group (ethylamine), eliminating the charge at physiological pH. In compound (4), conjugation of the acid to ethylaminediamine resulted in a conjugate with a free amino group, imparting a cationic charge. Although modifications of the aldehyde did not affect the anionic group on the glucuronic acid carboxyl, this modification did affect the overall charge of the QS-21 molecule. Compound (6) is zwitterionic, with negative charges on the glucuronic acid and a positive charge on the secondary amine formed on the aldehyde, whereas compound (7) is positively charged overall. Hydrophobicity changes (assessed by relative retention time to QS-21 on reversed-phase HPLC) were determined to be minimal and were within the range encompassed by naturally occurring adjuvant-active saponins such as QS-7 (relative retention time 0.72).

Antibody stimulation

All derivatives were tested for activity in stimulating antigen-specific antibody to OVA in C57BL/6 mice. Mice received three immunizations with OVA and 10 μg of QS-21 (1) or derivatives (2)–(4) (modified at the glucuronic acid carboxyl group) or (5)–(7) (modified at the aldehyde at triterpene carbon 4). Anti-OVA IgG1, $IgG2_b$, and IgG1 titers were determined by EIA (*Figure 2*). Unmodified QS-21 induced significant increases in IgG1, $IgG2_b$, and $IgG2_a$. Derivatives (2) and (3) also induced significant increases in antibody of all three isotypes although to a slightly lesser extent than QS-21. No adjuvant effect was noted for derivative (4) except for a 10-fold increase in $IgG2_b$. The antibody profile induced by OVA formulations adjuvanted with derivatives (5)–(7) was similar to that induced by the OVA/

Table 1 Characterization of QS-21 derivatives

Derivative	Blocking group	Reaction site	Theoretical molecular formula (formula weight)[a]	m/z of pseudo-molecular ions (relative intensity)[b]	Assignment of FAB-MS ion peaks	Relative retention time	Theoretical charge at physiological pH
(1)	None	None	$C_{92}O_{46}H_{148}$ (M = 1988.9)	2012 (100%)	$(M+Na)^+$	1.00	-
(2)	Glycine	Glucuronic acid carboxyl	$C_{94}O_{47}N_1H_{151}$ (M = 2045.9)	2085 (100%) 2069 (85%)	$(M+K)^+$ $(M+Na)^+$	0.97	-
(3)	Ethylamine	Glucuronic acid carboxyl	$C_{94}O_{45}N_1H_{153}$ (M = 2016.0)	2039 (100%)	$(M+Na)^+$	1.08	0
(4)	Ethylenediamine	Glucuronic acid carboxyl	$C_{94}O_{45}N_2H_{154}$ (M = 2031.0)	2054 (100%)	$(M+Na)^+$	0.80	+
(5)	Glycine	Aldehyde	$C_{94}O_{47}N_1H_{153}$ (M = 2048.0)	2071 (100%)	$(M+Na)^+$	0.93	-
(6)	Ethylamine	Aldehyde	$C_{94}O_{45}N_1H_{155}$ (M = 2018.0)	2019 (100%)	$(M+H)^+$	0.90	0
(7)	Ethylenediamine	Aldehyde	$C_{94}O_{45}N_2H_{156}$ (M = 2033.0)	2034 (100%)	$(M+H)^+$	0.78	+

[a]Theoretical formula weight was calculated from the exact mass of the commonest isotope of each element. [b]The m/z values for the most predominant peaks noted in fast atom bombardment-mass spectra are reported. The relative intensities of these peaks (expressed as % of the intensity of the most predominant peak) are reported in parentheses. The most predominant peak for QS-21 and most derivatives was the sodium adduct $(M+Na)^+$

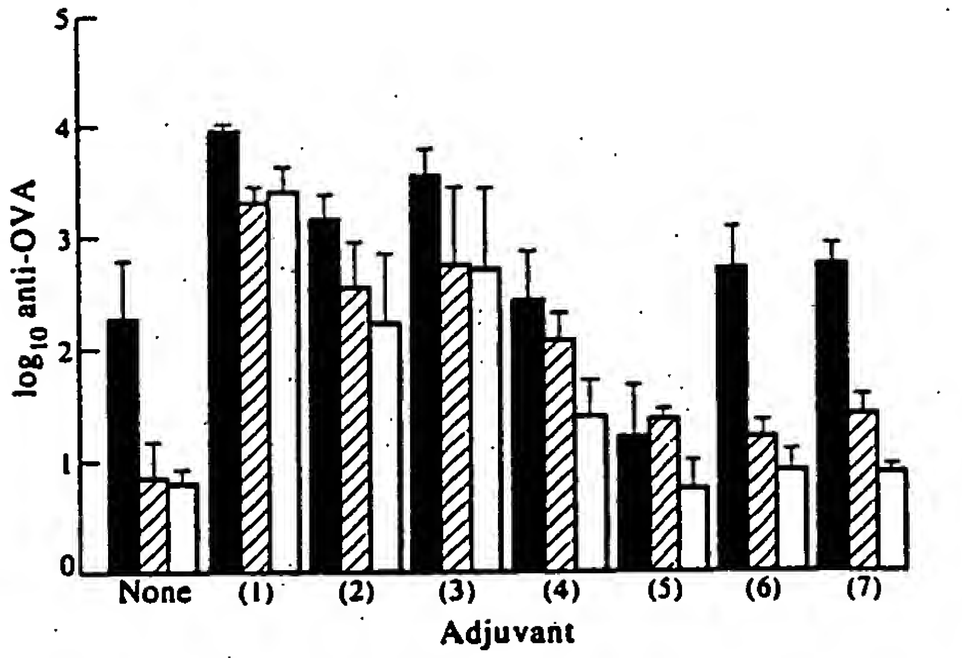

Figure 2 Antigen-specific antibody stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10 and 12 weeks of age with test formulations containing 25 μg OVA adjuvanted with 10 μg QS-21 or QS-21 derivative. A control group immunized with OVA in saline was included. Serum were collected one week after the third immunization and analyzed for anti-OVA of the IgG subclasses IgG1 (solid bars), $IgG2_b$ (cross-hatched bars), and $IgG2_a$ (stippled bars). Data are expressed as the mean and 1 standard error of the log 10 titer of the five mice in each group

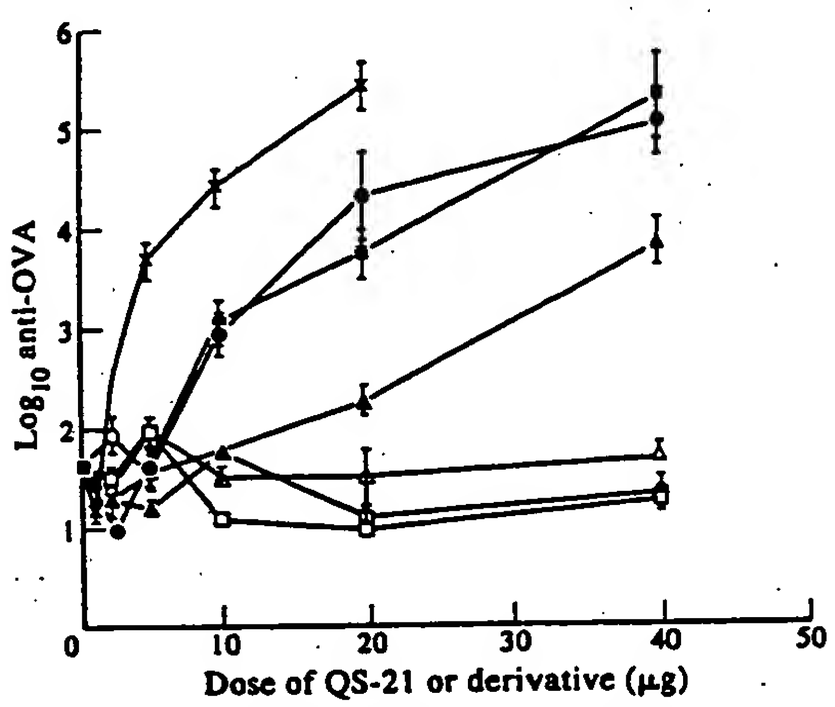

Figure 3 Effect of QS-21 derivative dose on antibody stimulation. C57BL/6 mice (10 per group) were immunized subcutaneously with 25 μg OVA and the indicated dose of QS-21 or QS-21 derivative in a total volume of 0.2 ml saline at 8 and 10 weeks of age. Sera were collected 1 week after the second immunization and an equivolume pool was made from each of the mice in a group; these pools were analyzed in quadruplicate by EIA on plates coated with OVA. Data are reported as the mean and 1 standard error of $\log_{10}$ titer. Test adjuvant:(1), X; (2), ●; (3), ■; (4), ▲; (5), ○; (6), □; (7), △

saline formulation, suggesting that these derivatives were inactive as adjuvants. The total IgG titer (not shown) also indicated the same trend.

The anti-OVA total IgG response to different doses of derivatives were compared in *Figure 3*. Mice were immunized subcutaneously with test vaccines containing 25 μg OVA and doses of QS-21 or derivative ranging between 2.5 and 40 μg. Serum was collected at 1 week after a second immunization and tested for total IgG to OVA. QS-21 stimulated anti-OVA IgG at doses between 5 and 10 μg, with some partial effect observed at 2.5 μg. A higher dose was required for QS-21 derivatives which were modified at the glucuronic acid carboxyl ((2)–(4)). However, despite the increase in minimum effective dose, all derivatives modified at the carboxyl retained the function of antibody stimulation. In addition to total IgG, antigen-specific IgG1, $IgG2_b$, and $IgG2_a$ were measured for these derivatives and were observed to increase according to the same dose response curves as for the total IgG (data not shown). In contrast, none of the derivatives prepared by conjugation to the triterpene aldehyde ((5)–(7)) stimulated any increase in antibody titer despite doses of up to 40 μg (approximately 16-fold higher than the lowest dose of QS-21 (2.5 μg) that stimulates any significant increase in titer). Hence, modification of the triterpene aldehyde of QS-21 effectively eliminated its property of antibody stimulation in this dose range, although activity at a higher dose could not be ruled out.

Class I-restricted cytotoxic T-lymphocyte stimulation

One of the more unique properties of QS-21 adjuvant is its activity for stimulation of MHC class I-restricted CTL in response to subunit vaccines. Hence, the derivatives were tested for stimulation of CTL to determine whether modification affects this property. The results are summarized in *Figure 4*. An OVA-transfected syngeneic cell (E.G7-OVA) was used as target. Splenocytes (from mice receiving 3 immunizations with test vaccines) were stimulated by mitomycin-C treated E.G7-OVA to induce CTL maturation; these splenocytes were then used as effector cells in the lysis assay (panel A). The specific killing induced by effector cells from mice receiving derivatives as adjuvants were compared to effectors from mice receiving QS-21 and was used as a measure of precursor CTL induced by these adjuvants. Additionally, denatured OVA was tested as an antigen stimulus to determine whether the derivatives induced an APC population with capacity for antigen processing (panel B).

Derivative (3) (prepared by modification of the glucuronic acid carboxyl with ethylamine) was as effective as QS-21 in stimulating precursor CTL that could be stimulated to mature by stimulation with mitomycin-C treated E.G7-OVA cells. Derivative (3) also induced precursor CTL that could be expanded by denatured OVA, although to a lesser extent than QS-21. Derivatives (2) and (4) (prepared by modification of the glucuronic acid carboxyl with glycine and ethylenediamine, respectively) also stimulated a CTL response. However, this response was lower than that induced by QS-21 and was observed only by use of processed antigen (mitomycin C-treated E.G7-OVA cells) as an antigen stimulus. Doses higher than 10 μg were not tested, so the possibility that these derivatives would induce a stronger lytic response at doses yielding maximum response in antibody stimulation cannot be ruled out. Derivatives (5)–(7) (prepared by modification of the triterpene aldehyde) were not active or poorly active in CTL induction, invoking responses that were similar to that induced by a nonadjuvanted OVA vaccine.

Effect of modification on detergent properties of QS-21

QS-21 and other saponins from *Quillaja saponaria* associate in micelles due to their amphipathic structure. As a measure of the propensity of these molecules to form micelles, the critical micellar concentration was determined (*Table 2*). The critical micellar concentration

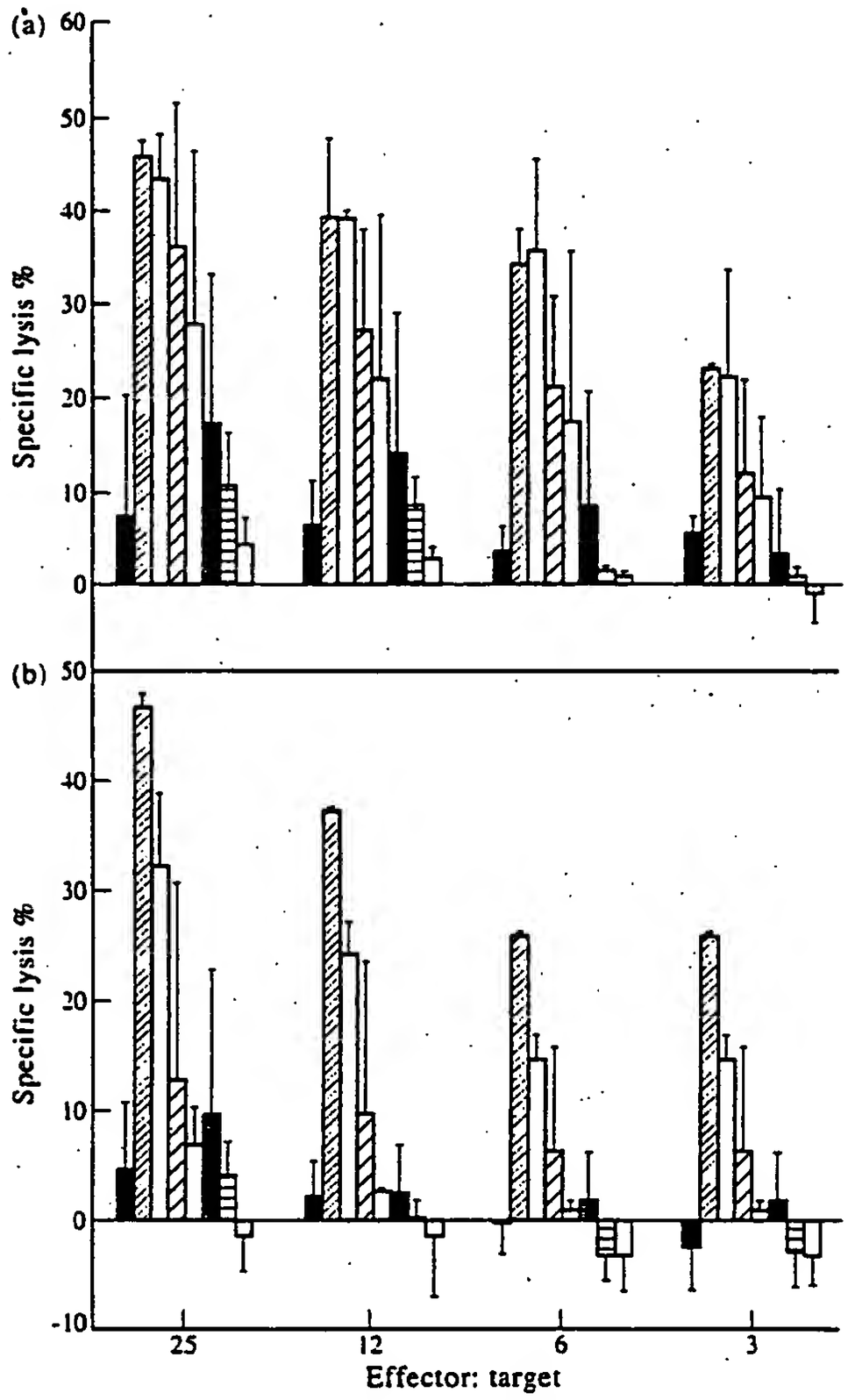

Figure 4 Cytotoxic T-lymphocyte stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10, and 12 weeks of age with test formulations containing 25 μg OVA adjuvanted with 10 μg QS-21 or QS-21 derivative. CTL activity was measured using E.G7-OVA cells and EL-4 cells as targets and splenic mononuclear cells from immunized animals as CTL effector cells. Splenic mononuclear cells were collected from 2 to 4 weeks after the last immunization. Maturation of precursor CTL to functional effector cells *in vitro* was carried out by specific antigen stimulation using mitomycin C-treated E.G7-OVA cells (panel A) and denatured OVA (panel B). Data are expressed as mean % specific lysis +1 standard deviation from two separate assays of pooled splenocytes from 2 or 3 mice after subtraction of background lysis of EL4 cells. Test adjuvant: None ▨; QS-21, (1), ▨; (3), ▨; (4), ▨; (2), □; (6), □; (7), ▨; (5), □

was minimally affected by modification of the glucuronic acid, with these derivatives having lower cmc values than the intact QS-21. This was consistent with the site of modification, which is in one of the hydrophilic domains of the QS-21 molecule. In contrast, the cmc values of the derivatives modified at the aldehyde were substantially higher than that of QS-21, ranging from 2.3-fold higher for the cationic ethylenediamine derivative to almost sixfold higher for the anionic glycine derivative, consistent with modification of QS-21 in one of the two hydrophobic domains expected to contribute to association.

The lysis of red blood cells in an *in vitro* assay was used as a second measure of detergent activity. The relative hemolytic activity of the derivatives was measured and compared to that of unmodified QS-21

Table 2 Detergent properties of QS-21 derivatives

Derivative	Critical micellar concentration (μM)[a]	Concentration inducing 50% hemolysis (μM)[b]
QS-21 (1)	26.6±4.9	4
(2)	22.0±7.7	16
(3)	16.0±2.2	7
(4)	13.3±8.2	17
(5)	147±2.1	>244[c]
(6)	89.6±0.9	>248
(7)	61.0±8.2	>246

[a]The critical micellar concentration was determined in phosphate-buffered saline, pH 7.0, as described in Materials and Methods. Data is expressed as mean ±1 standard deviation in assay of two separate preparations of each derivative. [b]The concentration inducing 50% hemolysis of sheep erythrocytes in an *in vitro* assay was determined as described in Materials and Methods. [c]Highest concentration tested

(*Table 2*). Modifications made at the glucuronic acid did not substantially affect the hemolytic activity. However, modifications at the triterpene aldehyde eliminated hemolytic activity up to the highest concentration tested (244–248 μM). The results were consistent with the results from the critical micellar concentration determination, indicating that the lysis of cellular membranes was affected by modifications that increased the cmc. For QS-21 and those derivatives that retained hemolytic activity, minimum hemolytic concentrations were lower than the cmc, indicating that the monomeric form of QS-21 and derivatives is the form which associates with the erythrocyte membrane.

DISCUSSION

All derivatives were tested for adjuvant activity by determining their effects on both antibody response and cellular mediated response to determine whether these activities were affected equally or would be affected in an unequal fashion. Augmentation of antibody but not CTL or the converse would suggest that different QS-21 functional groups or regions are involved in these separate activities. However, derivatives prepared by modification at the glucuronic acid carboxyl were active for stimulation of both antibody and CTL; derivatives prepared by modification of the triterpene aldehyde were inactive for both responses. This does not rule out the possibility that these activities can be mapped to separate regions that we have not yet examined. For example, diphosphoryllipid A has both adjuvant and toxic properties, but the derivative monophosphoryllipid A retains adjuvant activity, but exhibits significantly lower toxicity than the parent molecule[18].

Some moderate association between detergent activity and adjuvant activity was noted in this study. This was most evident in the three derivatives which were modified at the triterpene aldehyde. This modification resulted in substantial increases in both the critical micellar concentration and in concentrations required for hemolytic activities, indicating that the modification of the apolar triterpene interferes with self-association and membrane association. This was correlated with a loss of adjuvant activity. By contrast, modification of glucuronic acid, which is part of the hydrophilic glycoside and is not expected to participate in self-association,

did not substantially affect the detergent properties of this molecule and adjuvant activity was retained at high doses. However, this correlation between loss of hemolytic activity and loss of adjuvant activity is different from our previous observation that a naturally occurring *Quillaja saponaria* saponin, QS-7, is not hemolytic up to 500 μg ml^{-1}, but is adjuvant active[3]. Derivative (3) (conjugation of ethylamine to the glucuronic acid) is more hydrophobic than QS-21, has a lower critical micellar concentration than QS-21, and has similar hemolytic activity. However, the minimum effective dose of (3) indicated by the dose response for stimulation of antibody was several fold higher than native QS-21. We have previously observed that QS-21 is active as an adjuvant below the critical micellar concentration[17]. Hence, the adjuvant properties of QS-21 are not necessarily associated with its lysis of cell membranes or its properties of self association or membrane association.

Studies with muramyldipeptide (MDP) derivatives such as MTP-PE suggest that increasing hydrophobicity does not substantially affect antibody stimulation, but improves cell-mediated responses[19]. Lipophilicity was also noted to be important for the adjuvant effect of nonionic block copolymer adjuvants[20]. However, lipophilic derivative (3) of QS-21 was not improved over QS-21 in either minimum dose and levels of antibody stimulation or induction of cell-mediated immune responses, measured in this study as induction of Class I restricted CTL. However, it did induce the highest CTL response of the derivatives, suggesting that an increase in lipophilicity does influence this function. Although most of the derivatives were apparently less hydrophobic than QS-21, being retained less tightly on reversed-phase HPLC, the range of retention times of these derivatives was encompassed by that of naturally occurring, adjuvant-active, polar saponins from *Quillaja saponaria* such as QS-7, QS-17 and QS-18, which stimulate antibody response in mice in the same dose range as QS-21[3,4]. Hence, we expected that any differences observed in biological function of these derivatives would be primarily due to blocking of the functional groups, with the polarity changes playing a relatively minor role in the differences.

The three derivatives prepared by conjugation of small molecules to the glucuronic acid carboxyl retained substantial adjuvant activity. In addition to stimulating total antigen-specific IgG titer, these derivatives were active in stimulating antigen-specific IgG1, IgG2$_b$ and IgG2$_a$, suggesting a stimulation of both Th1 and Th2 cells. The ability to stimulate IgG2$_b$ and IgG2$_a$ antibody has been noted previously for unmodified QS-21[3,4], suggesting that modification at the glucuronic acid does not affect the Th1-type response associated with QS-21. Furthermore, these derivatives were active as adjuvants for induction of MHC Class I-restricted precursor CTL. Immunization with antigen/QS-21 has been suggested to set up an activated macrophage population that is highly efficient in the presentation of exogenously provided antigen such as OVA to the Class I MHC pathway[21]. One of the derivatives appeared to retain this property. The effector cells from mice immunized with OVA and derivative (3), modified at the glucuronic acid with ethylamine, were stimulated to produce mature CTL after stimulation with denatured OVA, suggesting that this derivative retained the ability of the native QS-21 molecule for activation of this APC population for processing and presenting OVA antigen. Hence, derivatives modified at the glucuronic acid carboxyl retained the ability to set up the same type of immunological responses as the native QS-21. This was in spite of modifications that blocked the anionic carboxyl group with a neutral or cationic molecule. Hence, it is unlikely that this functional group is directly involved in adjuvant function. However, the increase in minimum effective dose with these derivatives relative to QS-21 suggests a potential steric hindrance of a site important to activity. It has been proposed that the glucuronic acid on *Quillaja* saponins prevents aggregation of immune-stimulating complexes (ISCOM) containing *Quillaja* saponin[22]. Because we were able to utilize the QS-21 derivatives as adjuvants in soluble form, we did not attempt to prepare ISCOM with these QS-21 derivatives to determine whether the elimination of the charge affected ISCOM formation.

The glucuronic acid site is a potential site for conjugation directly to antigen. Conjugation of muramyl dipeptide adjuvant directly to either luteinizing hormone-releasing hormone[23] or to coliphage MS-2 viral peptide coupled to a polymeric carrier[24] yielded highly immunogenic complexes in the absence of additional adjuvant. This strategy could also be used with QS-21, potentially decreasing the required amount of antigen and/or adjuvant in a vaccine formulation. We have already demonstrated that QS-21 can be directly coupled through an amide linkage through the glucuronic acid carboxyl to free amino groups on lysozyme, resulting in a 1:1 molar conjugate that induces higher antibody titers to lysozyme than free lysozyme and QS-21[4]. Additional studies are ongoing to analyze both antibody and CTL responses to a 1:1 molar covalent conjugate of OVA: QS-21.

In contrast to modification of glucuronic acid, the modification of the aldehyde at C4 on the QS-21 triterpene severely diminished adjuvant activity. All three derivatives modified at the aldehyde were inactive as adjuvants over a dose range 10-fold higher than the minimum dose of native QS-21 associated with some stimulatory effect (2.5 μg). Hence, this aldehyde may be critical to the adjuvant function. One possible mechanism involving the aldehyde might be the formation of a Schiff base with a free amino group on a cellular target to stabilize a cellular interaction. Stabilization of interaction of MHC Class II+ antigen-presenting cells and T$_h$ cells via Schiff base interaction between free amino groups on antigen-presenting cells and aldehyde on the T cells has been noted[25]. The inactivation of the QS-21 adjuvant function by blocking the aldehyde suggests that it may also be involved in a Schiff base interaction with a free amino group on the surface of an immune cell target. However, a direct Schiff base-stabilized interaction of QS-21 with a particular immune cell population has not yet been demonstrated. It was shown through *in vivo* and *in vitro* cell depletion and reconstitution studies that macrophages are critical for both induction of precursor CTL to QS-21/subunit antigen vaccines as well as being critical for antigen processing during CTL maturation[21]. Hence, macrophages may be an important site of action for QS-21. However, other immune cell populations such as T cells cannot be ruled

out. Additional studies are planned to further determine the site of action of QS-21. Upon determination of the immune cells that interact with QS-21, the possible interaction of the aldehyde with these cells will be explored.

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A Toll-like receptor recognizes bacterial DNA

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DNA from bacteria has stimulatory effects on mammalian immune cells[1–3], which depend on the presence of unmethylated CpG dinucleotides in the bacterial DNA. In contrast, mammalian DNA has a low frequency of CpG dinucleotides, and these are mostly methylated; therefore, mammalian DNA does not have immuno-stimulatory activity. CpG DNA induces a strong T-helper-1-like inflammatory response[4–7]. Accumulating evidence has revealed the therapeutic potential of CpG DNA as adjuvants for vaccination strategies for cancer, allergy and infectious diseases[8–10]. Despite its promising clinical use, the molecular mechanism by which CpG DNA activates immune cells remains unclear. Here we show that cellular response to CpG DNA is mediated by a Toll-like receptor, TLR9. TLR9-deficient ($TLR9^{-/-}$) mice did not show any response to CpG DNA, including proliferation of splenocytes, inflammatory cytokine production from macrophages and maturation of dendritic cells. $TLR9^{-/-}$ mice showed resistance to the lethal effect of CpG DNA without any elevation of serum pro-inflammatory cytokine levels. The *in vivo* CpG-DNA-mediated T-helper type-1 response was also abolished in $TLR9^{-/-}$ mice. Thus, vertebrate immune systems appear to have evolved a specific Toll-like receptor that distinguishes bacterial DNA from self-DNA.

The Toll-like receptor (TLR) family is a phylogenetically conserved mediator of innate immunity that is essential for microbial recognition[11]. Mammalian TLRs comprise a large family with extracellular leucine-rich repeats (LRRs) and a cytoplasmic Toll/interleukin (IL)-1R (TIR) homology domain. So far, six members (TLR1–6) have been reported[12–14], and two additional members have been deposited in GenBank as TLR7 and TLR8 (accession numbers AF240467 and AF246971, respectively). TLR2 and TLR4 are responsible for immune responses to peptidoglycan (PGN) and lipopolysaccharide (LPS), respectively[15–22].

By using a BLAST search, we identified an expressed sequence tag (EST) clone (AA273731; mouse) that showed high similarity with the previously identified TLRs. Using this fragment as a probe, we isolated a full-length complementary DNA from the mouse macrophage cDNA library. We also isolated the human counterpart. Sequence analysis revealed the presence of regions conserved in the TLR family, such as LRR and TIR domain (Fig. 1a, b). Therefore, we designated this gene TLR9. Northern blot analysis of various tissues indicated that mouse TLR9 transcripts were most abundantly expressed in the spleen (Fig. 1c).

To assess the biological function of TLR9, we generated $TLR9^{-/-}$ mice by homologous recombination in embryonic stem (ES) cells. The targeting vector was constructed to replace a 1.0-kb fragment of the mouse *Tlr9* gene encoding a part of LRR with a neomycin resistance cassette (*neo*) (Fig. 2a). Correctly targeted ES cell clones were micro-injected into C57BL/6 blastocysts, which contributed to transmission of the mutated allele through the germ line. We intercrossed heterozygotes to produce offspring that were homozygous for the disrupted *Tlr9* allele (Fig. 2b). The mutant mice were

born at the expected mendelian ratio. We then investigated expression of TLR9 messenger RNA in the spleen by northern blot analysis. When we used the carboxy-terminal fragment as a probe, we detected *Tlr9* transcripts from the mutant mice at almost the same size but in reduced amounts compared with those from wild-type mice (Fig. 2c). We next carried out polymerase chain reaction with reverse transcription (RT–PCR) using spleen mRNA from the mutant mice. Sequence analysis of these products showed that the transcribed *Tlr9* gene contained the *neo* gene. The insertion of *neo* resulted in an appearance of a stop codon at the amino-terminal portion of TLR9, indicating that a functional TLR9 protein was not expressed in the mutant mice (Fig. 2d). $TLR9^{-/-}$ mice showed no abnormal composition of lymphocytes as determined by flow cytometry (data not shown).

MyD88 is an adaptor molecule involved in the signalling through the IL-1R and TLR families. We previously showed that MyD88 is essential for the response to IL-1, IL-18, LPS and many other bacterial cell-wall components[23]. We have also shown that the responses to CpG DNA are dependent on MyD88 and TRAF6 (ref. 24). $MyD88^{-/-}$ mice did not respond to CpG DNA, whereas both $TLR2^{-/-}$ and $TLR4^{-/-}$ mice responded normally to CpG DNA. These data indicate that CpG DNA is recognized by TLRs other than TLR2 and TLR4. Therefore, we analysed responses of $TLR9^{-/-}$ mice to CpG DNA. We first investigated the proliferation of splenocytes in response to CpG DNA (Fig. 3a). CpG DNA, but not non-CpG DNA induced proliferation of wild-type splenocytes in a dose-dependent manner. In contrast, $TLR9^{-/-}$ splenocytes did not proliferate in response to either CpG DNA or non-CpG DNA, although they showed a similar proliferative response to LPS as the wild-type cells. Wild-type B cells showed enhanced surface expression of major histocompatibility complex (MHC) class II in response to CpG DNA; however, a CpG-DNA-induced increase in MHC class II expression was not observed in $TLR9^{-/-}$ B cells (data not shown). These data indicate that $TLR9^{-/-}$ B cells are defective in

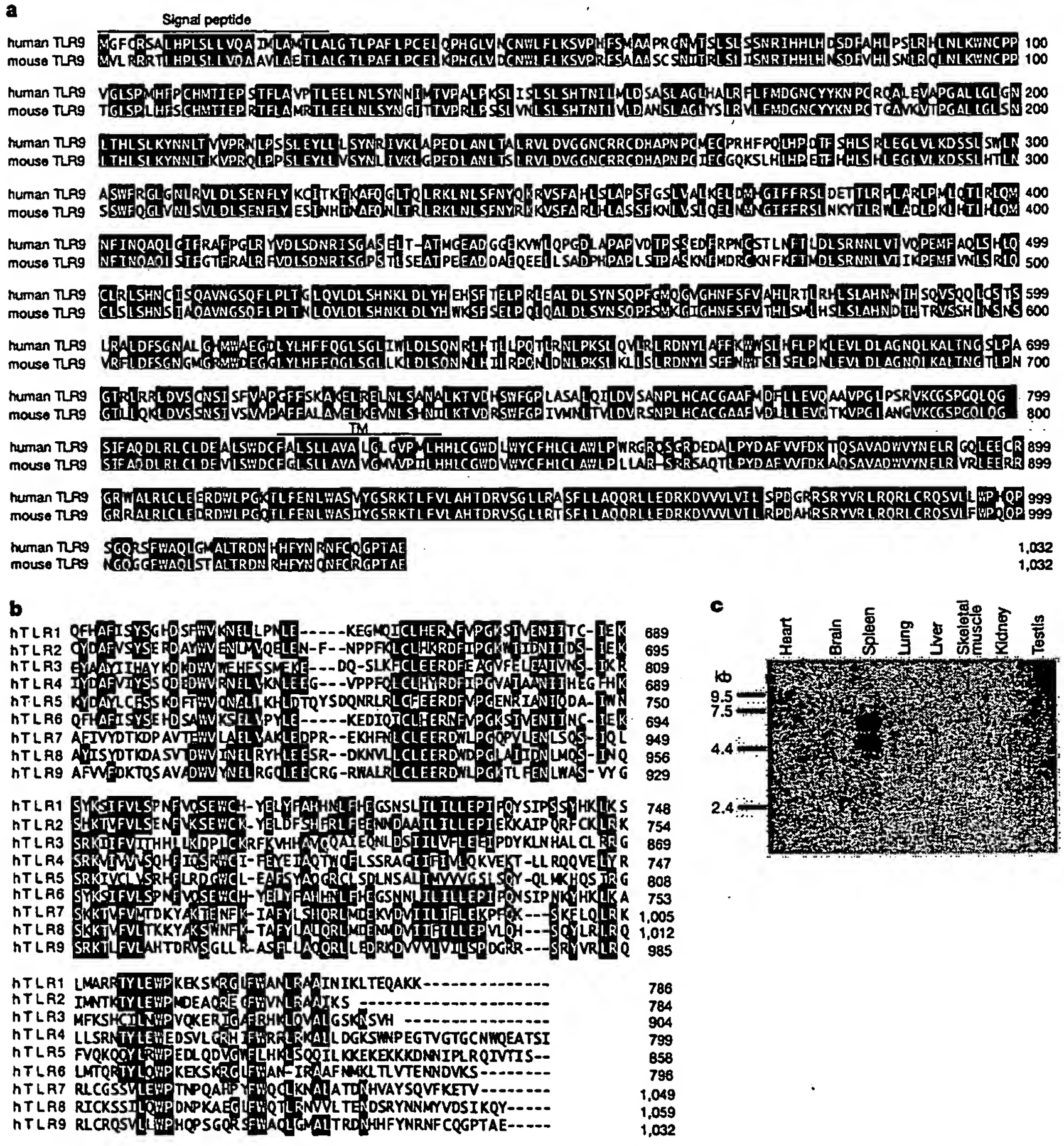

Figure 1 Amino-acid sequences and tissue distribution of TLR9. **a**, Alignment of human and mouse TLR9. Identical amino acids are indicated by a solid box. Human and mouse TLR9 share an overall amino-acid identity of 75.5%. The predicted signal peptide (human and mouse, residues 1–25) and transmembrane segments (TM; human, 819–836; mouse, 820–837) are indicated. During the preparation of this manuscript, the sequences for human TLR9 were deposited in GenBank by two other groups (accession numbers NM017442 and AF245704). **b**, Alignment of the cytoplasmic domains of human TLR family members. Amino-acid residues conserved in least four molecules are highlighted by solid boxes. **c**, A mouse multiple-tissue northern blot (Clontech) containing 2 μg of poly(A)$^+$ RNA was probed with a mouse TLR9 cDNA fragment.

their response to CpG DNA.

Next, we measured production of inflammatory cytokines from peritoneal macrophages by enzyme-linked immunoabsorbent assay (ELISA; Fig. 3b). Macrophages from wild-type mice produced tumour necrosis factor (TNF)-α, IL-6 and IL-12 in response to CpG DNA. The production was further enhanced when stimulated with a combination of interferon (IFN)-γ and CpG DNA. However, macrophages from TLR9$^{-/-}$ mice did not produce any detectable levels of inflammatory cytokines in response to CpG DNA even in the presence of IFN-γ. Macrophages from wild-type and TLR9$^{-/-}$ mice produced similar amounts of TNF-α, IL-6 and IL-12 in response to LPS, PGN, lipoprotein from *Escherichia coli*, Zymosan and whole heat-killed *Staphylococcus aureus* (Fig. 3b; and data not shown), indicating that TLR9$^{-/-}$ macrophages are specifically defective in their response to CpG DNA.

CpG-containing bacterial DNA is a potent stimulant for dendritic cells (DCs) to support T-helper type-1 (Th1) cell development[4–7]. Therefore, we examined CpG-DNA-induced cytokine production and upregulation of surface molecules in bone-marrow-derived murine DCs. Wild-type DCs produced IL-12 in response to CpG DNA; however, TLR9 $^{-/-}$ DCs did not produce any detectable levels of IL-12 (Fig. 3c). Wild-type DCs showed enhanced surface expression of CD40, CD80, CD86 and MHC class II when stimulated with CpG DNA. TLR9 $^{-/-}$ DCs did not show any enhanced expression of the surface molecules in response to CpG-DNA (Fig. 3d). Both wild-type and TLR9$^{-/-}$ DCs exhibited similar responses to LPS. Together, these findings indicate that TLR9 is essential for cellular responses to CpG DNA.

Signalling through TLRs occurs through the sequential recruitment of the adaptor molecule MyD88 and the serine/threonine kinase IRAK, and subsequently activates mitogen-activated protein (MAP) kinases and the nuclear factor NF-κB[23]. We next analysed activation of the intracellular signalling cascade in response to CpG DNA. In wild-type macrophages, stimulation with CpG DNA increased the DNA-binding activity of NF-κB, as determined by electrophoretic mobility shift assay (EMSA; Fig. 3e); however, NF-κB activity was not increased in response to CpG DNA in TLR9 $^{-/-}$ macrophages. LPS stimulation of TLR9$^{-/-}$ macrophages led to activation of NF-κB to the same extent as that of wild-type cells, indicating that CpG-DNA-induced activation of NF-κB was impaired in TLR9$^{-/-}$ macrophages. *In vitro* kinase assay showed that CpG DNA activated c-Jun N-terminal kinase (JNK) and IRAK in wild-type macrophages. Activation of both kinases was completely abolished in TLR9$^{-/-}$ macrophages (Fig. 3f, g). Thus, CpG-DNA-mediated signal transduction is dependent on TLR9.

Finally, we addressed the *in vivo* response to CpG DNA in TLR9$^{-/-}$ mice. CpG DNA can induce lethal shock in D-galactosamine (D-GalN)-sensitized mice[25]. Wild-type mice died within 12 h after D-GalN plus CpG DNA administration with marked elevation of serum concentrations of TNF-α, IL-6 and IL-12 (Fig. 4a, b). In contrast, all TLR9$^{-/-}$ mice survived without any increase in serum concentration of these inflammatory cytokines. Thus, TLR9$^{-/-}$ mice were highly resistant to CpG-DNA-induced shock syndrome. *In vivo* administration of CpG DNA has also been shown to induce a Th1-biased response[26]. CpG DNA and ovalbumin (OVA) were injected into the footpads, and lymph node cells were isolated at the 7-day time point and stimulated with OVA. The popliteal lymph node of

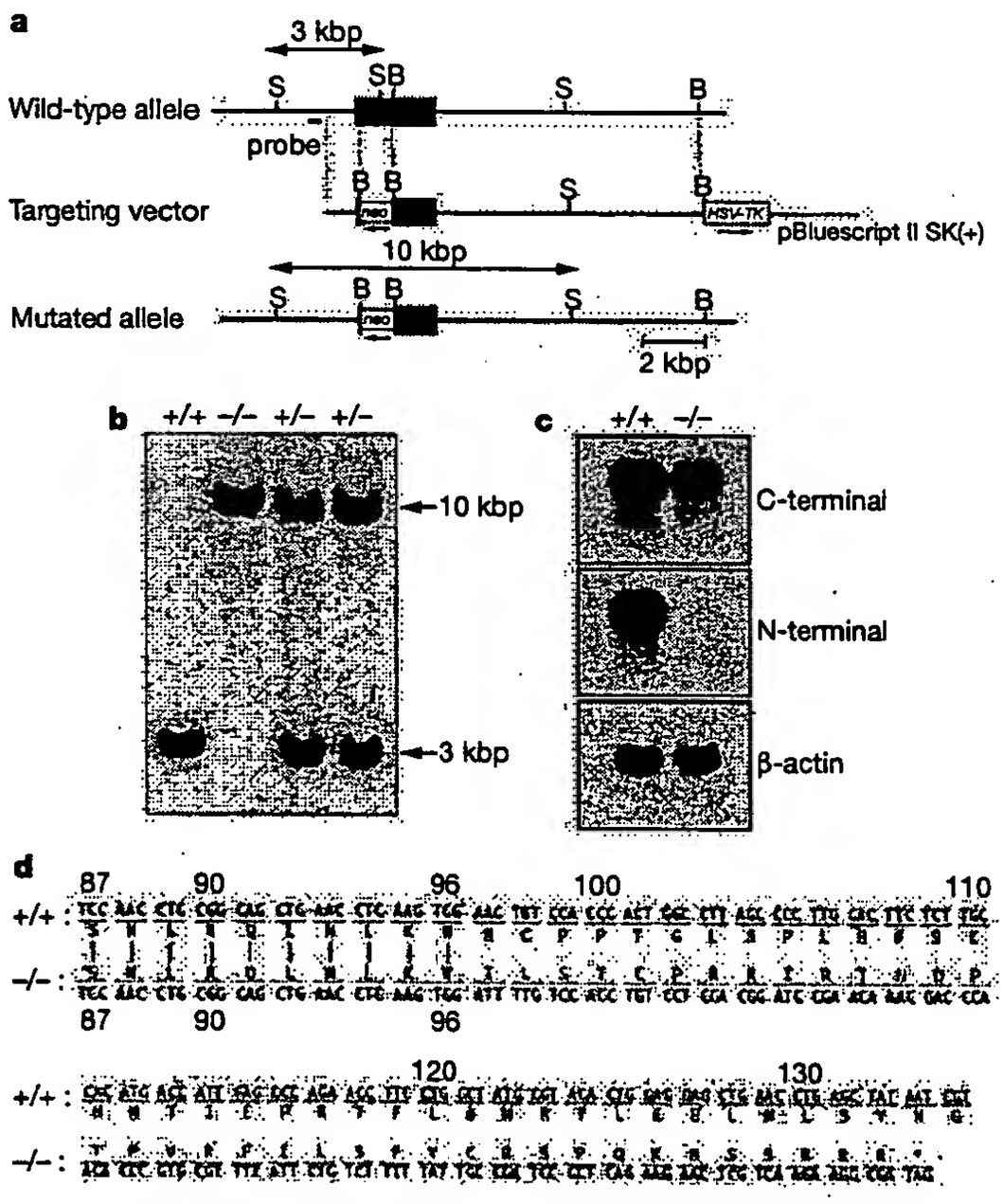

Figure 2 Targeted disruption of the mouse *Tlr9* gene. **a**, Maps of the TLR9 genome, the targeting vector and the predicted disrupted gene. Filled boxes denote the coding exon. Restriction enzymes: B, *Bam*HI; S, *Sca*I. **b**, Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with *Sca*I, electrophoresed and hybridized with the radiolabelled probe indicated in **a**. Southern blotting gave a single 3.0-kb band for wild-type (+/+), a 10-kb band for homozygous (−/−) and both bands for heterozygous mice (+/−). **c**, Northern blot analysis of splenocytes. Total RNA (10 μg) extracted from splenocytes was electrophoresed, transferred to a nylon membrane, and hybridized using the TLR9 C-terminal or N-terminal fragment as a probe. The same membrane was rehybridized with a β-actin probe. **d**, Comparison of predicted amino-acid sequences between wild-type (+/+) and TLR9$^{-/-}$ (−/−) cDNA. The numbers indicate predicted amino-acid position of wild-type TLR9.

CpG DNA-treated wild-type mice showed an increase in size compared with that of PBS treated mice, whereas TLR9$^{-/-}$ mice did not show any lymphadenopathy (data not shown). Lymph node cells from CpG-DNA-treated wild-type mice produced IFN-γ in response to OVA (Fig. 4c). In contrast, production of IFN-γ from TLR9$^{-/-}$ lymph node cells was not observed. Thus, CpG-DNA-induced Th1-like response was not observed in TLR9$^{-/-}$ mice.

The nature and localization of the CpG receptor are controversial. There is evidence that CpG DNA binds to cell-surface receptors that subsequently transduce stimulatory signals, because Sepharose beads coated with active CpG DNA stimulate B cells as free CpG DNA[27]. In contrast, other reports show that internalization of the DNA is required for activity[28]. Inhibitors of endosomal maturation such as bafilomaycin A or chloroquine abolish CpG-mediated cell activation, indicating that cellular uptake by nonspecific endocytosis and subsequent endosomal maturation precede cell activation[29,30]. Acidification of endosomal CpG DNA is coupled to the rapid generation of intracellular reactive oxygen species, followed by NF-κB activation[31]. Thus, in the latter case, it has been proposed that CpG DNA works through binding to an intracellular receptor. The presence of a transmembrane segment in the TLR9 gene strongly suggests that TLR9 is inserted into the membrane, but not present in the cytoplasm. Although the localization of TLR9 awaits assessment by immunostaining, confocal data show that tagged MyD88 colocalizes with tagged CpG DNA in endosomal structures, but not at the cell membrane (H.W., unpublished data). In contrast, LPS colocalizes with MyD88 at the cell membrane. This suggests that signalling is triggered by LPS at the cell membrane, whereas CpG DNA initiates signalling after translocation to endosomes. This assumption may well correlate with the finding that CpG-DNA-induced IRAK activation is delayed as compared with that stimulated with LPS (Fig. 3g). The identification of CpG DNA signalling receptor will pave the way to understanding the mechanism by which CpG DNA is recognized as well as by which the recognition of CpG DNA is translated into a strong Th1 response. Furthermore, TLR9$^{-/-}$ mice will provide a useful model for clarifying to what extent recognition of CpG DNA contributes to host immune responses against bacterial infections. □

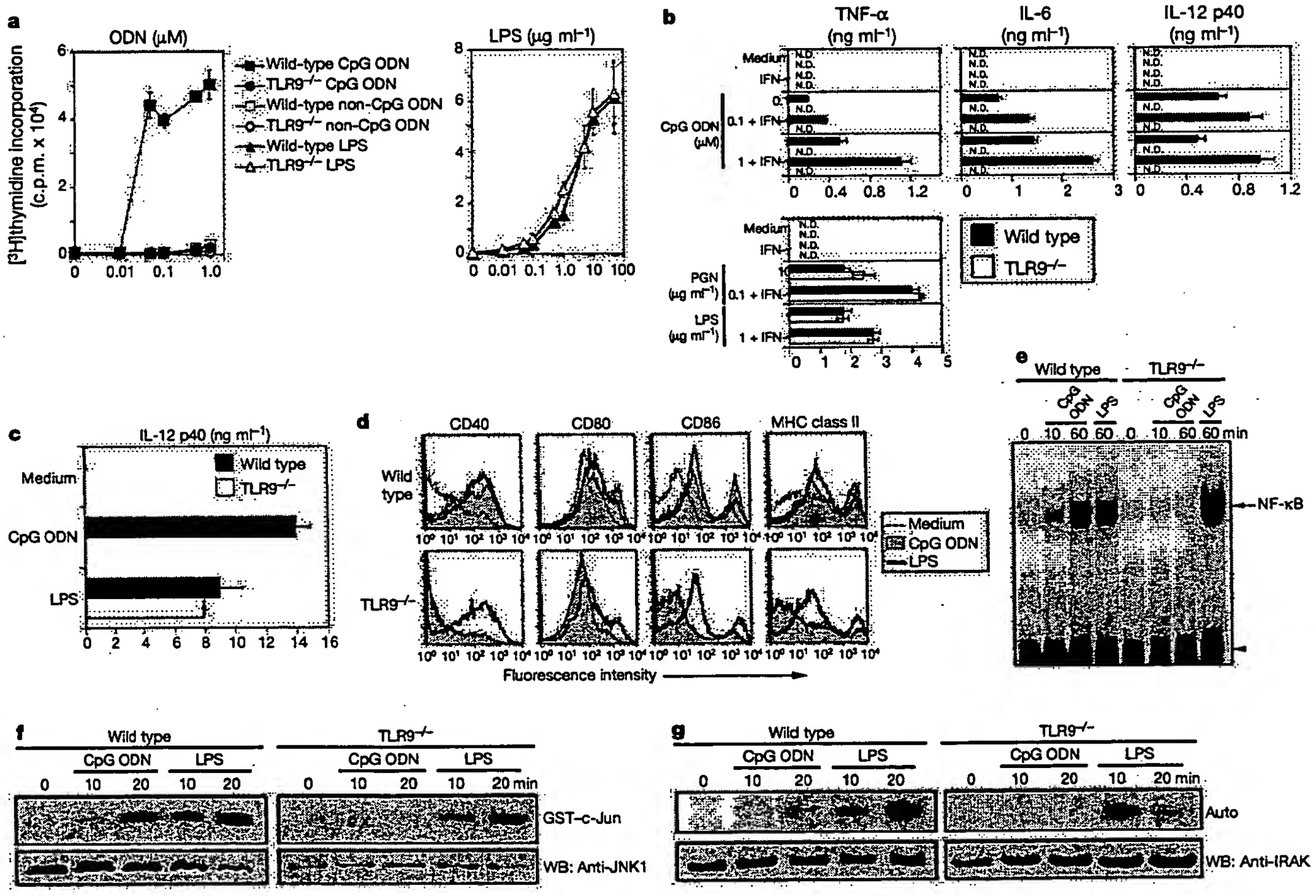

Figure 3 Impaired responses to CpG ODN in TLR9$^{-/-}$ cells. **a**, Splenocytes from wild-type or TLR9$^{-/-}$ mice were cultured with the indicated concentrations of CpG ODN, non-CpG ODN or LPS for 48 h plus pulsed [^{3}H]thymidine for the last 8 h. [^{3}H]thymidine incorporation was measured by β-scintillation counting. Data indicate mean ± s.d. of triplicate samples of one representative experiment. **b**, Peritoneal macrophages from wild-type or TLR9$^{-/-}$ mice were stimulated with CpG ODN (0.1 or 1.0 μM), PGN (10 μg ml^{-1}) or LPS (1.0 μg ml^{-1}) in the presence or absence of 30 U ml^{-1} IFN-γ for 24 h, and concentrations of TNF-α, IL-6 and IL-12 p40 in the culture supernatants were measured by ELISA. Similar results were obtained from three independent experiments. Data indicate mean ± s.d. N.D., not detected. **c**, Wild-type or TLR9$^{-/-}$ dendritic cells (DCs) derived from bone marrow were cultured with CpG ODN or LPS. Concentrations of IL-12 p40 in culture supernatants were measured by ELISA. Data indicate mean ± s.d. **d**, DCs were stimulated with CpG ODN or LPS for 48 h and analysed for cell-surface expression of the indicated molecules by flow cytometry. **e**, Peritoneal macrophages from wild-type and TLR9$^{-/-}$ mice were stimulated with 1.0 μM CpG ODN or 1.0 μg ml^{-1} LPS for the indicated durations. NF-κB activity was determined by EMSA. Arrow indicates the inducible NF-κB complex; arrowhead indicates free probe. **f**, Peritoneal macrophages from wild-type and TLR9$^{-/-}$ mice were stimulated with 1.0 μM CpG ODN or 1.0 μg ml^{-1} LPS for the indicated durations. Cell lysates were prepared and immunoprecipitated with anti-JNK antibody. JNK activity was measured by *in vitro* kinase assay using a GST–c-Jun fusion as a substrate (top). The same lysates were blotted with anti-JNK (bottom). **g**, The same cell lysates in **f** were immunoprecipitated with anti-IRAK antibody. The kinase activity of IRAK was measured by *in vitro* kinase assay (top). The same lysates were blotted with anti-IRAK antibody (bottom). Auto, autophosphorylation.

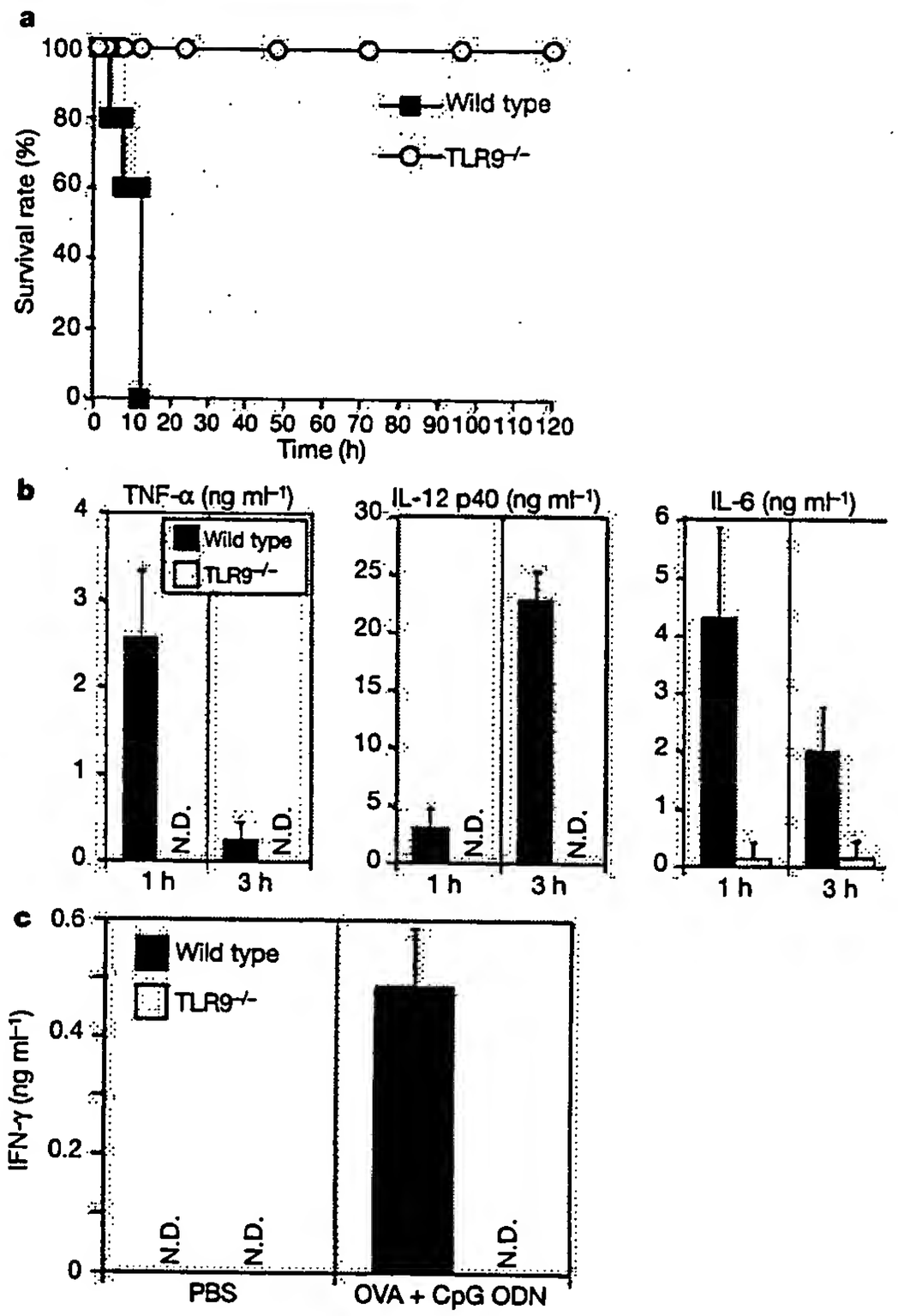

Figure 4 Resistance to CpG ODN-induced shock in TLR9$^{-/-}$ mice. **a**, Age-matched wild-type ($n = 5$) and TLR9$^{-/-}$ mice ($n = 5$) were intraperitoneally injected with CpG ODN (20 nmol) and D-GalN (20 mg). Survival was monitored for 5 d. **b**, Wild-type and TLR9$^{-/-}$ mice were intraperitoneally injected with CpG ODN and D-GalN. Sera were taken at 1 or 3 h after injection. Serum concentrations of TNF-α, IL-12 p40 and IL-6 were measured by ELISA. N.D., not detected. Results are mean of sera samples from three mice. **c**, Age-matched wild-type and TLR9$^{-/-}$ mice were injected with PBS or CpG ODN (5 nmol) plus OVA (150 μg) into the hind footpads. Popliteal lymph node cells were collected after 7 d and cultured with OVA for 24 h. Production of IFN-γ from lymph node cells was analysed by ELISA. Data indicate mean ± s.d. N.D., not detected.

Methods

Cloning of TLR9

A GenBank search resulted in identification of a mouse EST that has a significant similarity with human TLR4. Using PCR-amplified EST as a probe, a full-length cDNA clone containing the complete TLR9 open reading frame was isolated from the mouse RAW 264.7 cDNA library. Human genomic sequence that showed significant homology with the mouse TLR9 gene was found in GenBank. On the basis of this sequence, rapid amplification of cDNA ends protocol was performed to isolate full-length cDNA from U937 cDNA library.

Generation of TLR9$^{-/-}$ mice

The TLR9 genomic DNA was isolated from 129/Sv mouse genomic library and characterized by restriction enzyme mapping and sequencing analysis. The targeting vector was constructed by replacing a 1.0-kb fragment encoding a part of LRR region with a neomycin-resistance gene cassette (*neo*), and a herpes simplex virus thymidine kinase driven by MC1 promoter was inserted into the genomic fragment for negative selection (Fig. 2a). The targeting vector was transfected into embryonic stem cells (E14.1). G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and southern blotting. Homologous recombinants were micro-injected into C57BL/6 blastocysts. Chimaeric mice were mated with C57BL/6 female mice, and heterozygous F_1 progenies were intercrossed in order to obtain TLR9$^{-/-}$ mice. All mice analysed here were F_1 progeny of 129/Ola × C57BL/6.

Reagents

Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN) (TCC-ATG-ACG-TTC-CTG-ATG-CT)[21] was purchased from TIB MOLBIOL or Hokkaido System Science. Phosphorothioate-stabilized non-CpG ODN (GCT-TGA-TGA-CTC-AGC-CGG-AA)[5] was purchased from Hokkaido System Science. LPS from *Salmonella minnesota* Re-595 and PGN from *Staphylococcus aureus* were purchased from Sigma and Fluka, respectively[21].

Measurement of cytokine production from macrophages

Thioglycollate-elicited peritoneal macrophages were cultured with the indicated concentrations of CpG ODN, LPS or PGN for 24 h. Concentrations of TNF-α, IL-6 and IL-12 p40 in the culture supernatants were measured by ELISA.

Preparation and analysis of dendritic cells

Bone-marrow cells from wild-type or TLR9$^{-/-}$ mice were cultured with 10 ng ml^{-1} mouse granulocyte macrophage-colony stimulating factor (Peprotech) in RPMI1640 medium supplemented with 10% fetal bovine serum. At day 6, immature DCs were collected and cultured in the absence or presence of 0.1 μM CpG ODN or 0.1 μg ml^{-1} LPS in a fresh medium for a further 2 days. The concentration of IL-12 p40 in the culture supernatants was measured by ELISA. DCs were stained with biotinylated antibodies against CD40, CD80, CD86 or MHC class II, and developed with PE-conjugated streptavidin. Flow cytometric analysis was performed using a FACSCalibur with CELLQuest software (Becton Dickinson).

EMSA and *in vitro* kinase assay

Thioglycollate-elicited peritoneal macrophages (1×10^6 cells) from wild-type and TLR9$^{-/-}$ mice were stimulated for the indicated periods and then nuclear proteins were extracted. The extracts were incubated with a specific probe containing NF-κB DNA-binding sites, electrophoresed and visualized by autoradiography.

Thioglycollate-elicited peritoneal macrophages were stimulated with 1.0 μM CpG ODN or 1.0 μg ml^{-1} LPS for the indicated periods. JNK and IRAK activities in cell lysates were measured by *in vitro* kinase assays as described[23].

Cytokine production of presensitized lymph nodes

Age-matched wild-type and TLR9$^{-/-}$ mice were injected subcutaneously with CpG ODN (5 nmol) plus soluble OVA (150 μg) into the hind footpads. Seven days later, popliteal lymph nodes were collected and cultured with 100 μg ml^{-1} OVA for 24 h. Concentrations of IFN-γ in the culture supernatants were measured by ELISA.

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Structure of the bacteriophage ϕ29 DNA packaging motor

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Motors generating mechanical force, powered by the hydrolysis of ATP, translocate double-stranded DNA into preformed capsids (proheads) of bacterial viruses[1,2] and certain animal viruses[3]. Here we describe the motor that packages the double-stranded DNA of the *Bacillus subtilis* bacteriophage ϕ29 into a precursor capsid. We determined the structure of the head–tail connector—the central component of the ϕ29 DNA packaging motor—to 3.2 Å resolution by means of X-ray crystallography. We then fitted the connector into the electron densities of the prohead and of the partially packaged prohead as determined using cryo-electron microscopy and image reconstruction analysis. Our results suggest that the prohead plus dodecameric connector, prohead RNA, viral ATPase and DNA comprise a rotary motor with the head–prohead RNA–ATPase complex acting as a stator, the DNA acting as a spindle, and the connector as a ball-race. The helical nature of the DNA converts the rotary action of the connector into translation of the DNA.

The bacteriophage ϕ29 (Fig. 1) is a 19-kilobase (19-kb) double-stranded DNA (dsDNA) virus with a prolate head and complex structure[4]. The prohead (Fig. 1), into which the DNA is packaged, is about 540 Å long and 450 Å wide[5]. The ϕ29 connector, a cone-shaped dodecamer of gene product 10 (gp10), occupies the pentagonal vertex at the base of the prohead[5] and is the portal for DNA entry during packaging and DNA ejection during infection[6]. The connector, in association with the oligomeric, ϕ29-encoded prohead RNA (pRNA) and a viral ATPase (gp16), is required for DNA packaging[7–9]. However, only the first 120 bases of the 174-base pRNA are essential for packaging[7]. The covalent adduct of the genomic dsDNA with gp3 (DNA–gp3) can be packaged into proheads in about three minutes *in vitro* (P.J.J., unpublished results). The connector proteins of tailed phages[6] vary in relative molecular mass (M_r) from 36,000 (36K) in ϕ29 to 83K in phage P22, and assemble into oligomers with a central channel. The structure of the isolated ϕ29 connector has been studied by atomic force microscopy[10] and cryo-electron microscopy (cryo-EM) of two-dimensional arrays[11], immuno-electron microscopy[12] and X-ray crystallography[13,14].

The connector structure, as now determined by X-ray crystallography, can be divided into three, approximately cylindrical regions: the narrow end, the central part, and the wide end, having external radii (Å) of 33, 47 and 69, respectively (Fig. 2). These regions are respectively 25, 28 and 22 Å in height, making the total connector 75 Å long. The internal channel has a diameter of about 36 Å at the narrow end, increasing to 60 Å at the wide end. Comparison with electron microscopy reconstructions[5,11] shows that the narrow end protrudes from the portal vertex of the phage head, is associated with the multimeric pRNA, and binds the lower collar in the mature virus.

The electron density of the connector was interpreted in terms of the amino-acid sequence[15] and was confirmed by the two Hg sites (see Methods section) corresponding to the only cysteine residues in the sequence. Residues 1 to 11, 229 to 246, and 287 to 309 at the carboxy terminus are not seen in the electron density. The second and third disordered regions are both located on the inside of the channel, close to the junction of the central and wide regions. The structure is dominated by three long helices (α1, α3 and α5) in each monomer that run the length of the central region, joining the two end domains of the connector (Fig. 2). These helices are arranged at an angle of about 40° with respect to the central 12-fold axis. The two end domains are composed predominantly of β-sheets and extended polypeptide chains. Immuno-electron microscopy mapping of the sequence onto the connector surface[12] is consistent with the X-ray structure only as far as localization of the external amino-terminal residues with the pRNA-binding region at the narrow end of the connector. The RNA recognition motif structure, previously predicted for the N-terminal regions of the connector monomer[16,17], is not present in the structure.

The surface of the monomer presents a net negative charge to one neighbour and a net positive charge to the other neighbour, possibly aiding the assembly of the dodecamer. The exterior of the connector has no significant regions of charge accumulation, implying that its rotation might be facilitated by its oily, smooth, external surface. However, the basic character of the disordered 11 amino-terminal residues could alter the surface properties to some extent and may facilitate interaction with the pRNA. In contrast, the inside of the channel has a preponderance of negative charge at its wide end, which may repel the DNA, permitting its smooth passage during packaging and ejection. The channel through which messenger RNA is translocated in reoviruses has similar properties[18].

We have determined the structures of four distinct types of ϕ29

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INNATE IMMUNITY-STIMULATING COMPOSITIONS OF CPG AND SAPONIN AND METHODS THEREOF

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CROSS-REFERENCE TO RELATED APPLICATION

This U.S. Utility Patent Application claims priority from U.S. Provisional Application No. 60/200,853, filed May 1, 2000, U.S. Provisional Application No. 60/175,840, filed January 13, 2000 and U.S. Utility Patent Application No. 09/369,941, filed August 6, 1999, which claims benefit of U.S. Provisional Application No. 60/128,608, filed April 8, 1999, now abandoned, and U.S. Provisional Application No. 60/095,913, filed August 10, 1998, now abandoned.

FIELD OF THE INVENTION

The present invention is in the field of immune enhancers. The compositions of the invention stimulate innate immunity.

BACKGROUND OF THE INVENTION

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria* Molina. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. These saponins have been substantially purified by various methods including high pressure liquid chromatography ("HPLC"), low pressure liquid silica chromatography, and hydrophilic interactive chromatography ("HILIC").

The substantially pure saponins have been found to be useful as immune adjuvants when used with vaccine antigens for enhancing immune responses to such antigens in individuals. (Kensil, et al., U.S. Patent No. 5,057,540; Kensil, et al., *J. Immunol.* 148:2357 (1991); Marciani, et al., *Vaccine* 9:89 (1991).)

Recently, oligonucleotides containing the unmethylated cytosine-guanine ("CpG") dinucleotide in a particular sequence context or motif have been shown to be potent stimulators of several types of immune cells *in vitro*. (Weiner, et al., *Proc. Natl. Acad. Sci.* 94:10833 (1997).) An oligonucleotide comprising an unmethylated CpG motif within which is at least one unmethylated CpG dinucleotide has been shown to activate the immune system. CpG motifs can stimulate monocytes, macrophages, and dendritic cells that can produce several cytokines, including the T helper 1 ("Th 1") cytokine interleukin ("IL") 12. (Carson, et al., *J. Exp. Med.* 186:1621 (1997).) This effect causes the induction of IFN-γ secretion by natural killer (NK) cells, which in turn, activates macrophages and enhances immunoglobulin isotype switching to IgG2a, a hallmark of T helper Type 1 cell immunity and differentiation. (Chu, et al., *J. Exp. Med.* 186:1623 (1997).) Klinman, et al., have shown that a DNA motif consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines (GpA or ApA) and two 3′ pyrimidines (TpC or TpT) optimally stimulated B cells to produce IL-6 and IL-12, and stimulated CD4+ T cells to produce IL-6 and IFN-γ both *in vitro* and *in vivo*. (Klinman, et al., *Proc. Natl. Acad. Sci.*, 93:2879 (1996).) Davis, et al., discovered that nucleic acids containing at least one unmethylated CpG dinucleotide may affect the immune response of a subject (Davis, et al., WO 98/40100). Kensil, et al., previously showed that a combination of

QS-21 and CpG oligonucleotides have synergistic adjuvant activity for antigen-specific responses when combined with a vaccine antigen (Kensil, U.S.S.N. 09/369,941, the contents of which are fully incorporated by reference herein).

Recently, it has been shown that CpG administration, in the absence of a vaccine antigen, can protect a mouse against an otherwise lethal infection with an intracellular bacteria, such as *Listeria monocytogenes* or *Francisella tularensis*, if the CpG is administered between 2-3 days prior or no earlier than 2 weeks prior to the infection. (Elkins, et al., *J. Immunol.* 162: 2991 (1999).). This result suggests an activation of innate immunity. It has been hypothesized that CpG motifs are a danger signal that activate protective innate immune defenses (Krieg, et al., *J. Immunol.* 161: 2428 (1998)), in particular (NK) cell activity. CpG motifs appear to stimulate natural killer cell activity through direct CpG stimulation of natural killer cells or through natural killer-active cytokines secreted by CpG-stimulated monocytes.

SUMMARY OF THE INVENTION

Since innate immunity plays an important role in the protective response to infection with certain microbial agents and tumors, a need exists to characterize other agents that may safely stimulate innate immunity. Such agents may be potentially incorporated in future therapeutic agents. Surprisingly, a combination of an oligonucleotide comprising at least one unmethylated CpG dinucleotide and a saponin was found to be a powerful stimulator of natural killer cell activity compared to either compound alone. NK cell activity was significantly higher for a composition comprising a CpG-containing oligonucleotide/saponin combination compared to either

the saponin or the unmethylated CpG-containing oligonucleotide and represented a positive synergistic effect. Further, the saponin alone was shown to induce a higher natural killer cell response than the unmethylated CpG-containing oligonucleotide. Further, both the saponin alone and the combination of saponin/a CpG-containing oligonucleotide induced an innate immunity that enabled stronger protection against an infection than the CpG-containing oligonucleotide. Together, these results establish that a composition comprising saponin alone and a composition comprising an oligonucleotide comprising at least one unmethylated CpG dinucleotide plus a saponin are candidate compositions to induce innate immunity.

Accordingly, in a first aspect, the invention covers a composition comprising: (a) a saponin; and (b) an oligonucleotide comprising at least one unmethylated CpG dinucleotide. Preferably, the composition provides that the saponin is derived from *Quillaja saponaria*, and more preferably, the saponin is chemically modified or comprises a substantially pure saponin. In a preferred embodiment of the first aspect, the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21, and more preferably, the substantially pure saponin comprises QS-21. In yet other preferred embodiments of the first aspect, the composition is further directed to one in which the oligonucleotide is chemically modified. More particularly, the oligonucleotide is modified with at least one phosphorothioate internucleotide linkage. A preferred embodiment of the first aspect encompasses the composition wherein the oligonucleotide comprises a CpG motif having the formula $5'X_1CGX_2 3'$, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or

thymine, and X_2 is cytosine, thymine, or adenine. More preferably, the CpG motif comprises TCTCCCAGCGTGCGCCAT or TCCATGACGTTCCTGACGTT or TCGTCGTTTTGTCGTTTTGTCGTT. The composition, according to the first aspect of the invention, preferably increases an innate immune response when administered to a mammal or a human. Still another preferred embodiment is directed to the composition wherein the composition enhances a natural killer cell response, preferably in a positive synergistic manner.

In a second aspect, the invention is directed to a method for stimulating innate immunity comprising administering an effective amount of a composition comprising: (a) a saponin; and (b) an oligonucleotide comprising at least one unmethylated CpG motif to an individual. Preferably, the method provides that the saponin is derived from *Quillaja saponaria*, and more preferably, the saponin is chemically modified or comprises a substantially pure saponin. In a preferred embodiment of the second aspect, the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21, and more preferably, the substantially pure saponin comprises QS-21. In yet other preferred embodiments of the second aspect, the method is further directed to one in which the oligonucleotide is chemically modified. More particularly, the oligonucleotide is modified with at least one phosphorothioate internucleotide linkage. A preferred embodiment of the second aspect encompasses the method wherein the oligonucleotide comprises a CpG motif having the formula 5′X_1CG$X_2$3′, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. More preferably, the CpG motif comprises

TCTCCCAGCGTGCGCCAT or TCCATGACGTTCCTGACGTT or TCGTCGTTTTGTCGTTTTGTCGTT. The method, according to this second aspect of the invention, preferably further increases an innate immune response when administered to a mammal or a human. Still another preferred embodiment is directed to the method for further enhancing a natural killer cell response, preferably in a positive synergistic manner.

A third aspect of the invention provides for methods for stimulating innate immunity comprising administering an effective amount of a composition comprising a saponin only to an individual. Preferably, the method provides that the saponin is derived from *Quillaja saponaria*, and more preferably, the saponin is chemically modified or comprises a substantially pure saponin. In a preferred embodiment of the third aspect, the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21, and more preferably, the substantially pure saponin comprises QS-21. The method, according to the third aspect of the invention, preferably further increases an innate immune response when administered to a mammal or a human. Still another preferred embodiment is directed to the method for further enhancing a natural killer cell response.

DESCRIPTION OF THE FIGURES

Figure 1 is a graphic representation showing the enhancement of the natural killer cell response by QS-21 or by QS-21/CpG oligodeoxynucleotide (sequence 1826) combination, as evidenced by lysis of the NK-sensitive cell line YAC-1.

Figure 2 is a graphic representation showing the optimal timing of administration of QS-21/CpG oligodeoxynucleotide (sequence 1826) combination, as evidenced by lysis of the NK-sensitive cell line YAC-1.

Figure 3 is a graphic representation depicting the NK activating activity of QS-21, QS-7, or CpG oligodeoxynucleotide (sequence 1826), as evidenced by dose response curves for individual compounds for enhancing the NK cell response against the NK-sensitive cell line YAC-1.

Figure 4 is a graphic representation depicting the NK activating activity of various mixtures of QS-21, QS-7, and CpG oligodeoxynucleotides (sequences 1826 and 2006), as evidenced by lysis of the NK-sensitive cell line YAC-1.

Figure 5 is a graphic representation illustrating protection of Balb/c mice against an intraperitoneal challenge with 10^5 colonies of *Listeria monocytogenes* after administration of various formulations three days prior to challenge.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution and have hemolytic activity in most cases. The invention encompasses the saponin per se, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also embodies biologically active fragments thereof. The term "saponin" also encompasses chemically modified saponins.

The saponins of the present invention may be obtained from the tree *Quillaja*

saponaria Molina. (Dalsgaard, *Acta Veterinia Scandinavica*, 69:1 (1978).) A partially purified saponin enriched extract, prepared as described by Dalsgaard, ("Quil-A") has adjuvant activity. Such an extract can be further separated. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. (Kensil, et al., U.S. Patent No. 5,057,540.) These saponins have been substantially purified by various methods including HPLC, low pressure liquid silica chromatography, and HILIC.

"QS-21" designates the mixture of components QS-21-V1 and QS-21-V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 μm particle size, 300A pore, 4.6 mm ID x 25 cm length) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21-V1 and QS-21-V2 when describing experiments performed on the further purified components.

The present invention may also employ chemically modified saponins. According to Kensil, et al., U.S. Patent No. 5,443,829, the contents of which are fully incorporated by reference herein, such chemically modified saponins can be obtained in several ways. For example, the aldehyde group of either purified QS-17, QS-18, QS-21, or mixtures thereof, or purified fractions obtainable from *Quillaja saponaria* Molina bark and comprising QS-17, QS-18, and QS-21 can be reduced with a mild reducing agent, such as sodium or lithium borohydride, to give the corresponding alcohol. Alternatively, the aldehyde of QS-17, QS-18, and QS-21, mixtures thereof, or purified fractions obtainable from *Quillaja saponaria* Molina bark and comprising QS-17, QS-18,

and QS-21 can be subjected to reductive amidation with a primary amine and a reducing agent to give the corresponding amino derivative of QS-17, QS-18, and QS-21. According to Kensil, et al., U.S. Patent No. 5,583,112, the contents of which are fully incorporated by reference herein, the carboxyl group on the glucuronic acid of saponins from *Quillaja saponaria* Molina can be conjugated to a protein, a peptide, or a small molecule containing a primary amine. According to Higuchi, et al., *Phytochemistry* 26:229 (1987)), saponins from *Quillaja saponaria* may be deacylated by alkaline-catalyzed hydrolysis. According to Marciani, et al., U.S. Patent No. 5,977,081, the contents of which are fully incorporated by reference herein, the carboxyl group on the glucuronic acid of nonacylated or deacylated saponins from *Quillaja saponaria* may be conjugated to a lipid, fatty acid, polyethylene glycol, or terpene. Thus, the present invention relates to a chemically modified saponin or a biologically active fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract. Adjuvant-active saponins and adjuvant-inactive saponins fall within the scope of the invention described herein provided that these saponins stimulate innate immunity alone or in combination with a CpG dinucleotide.

In other embodiments of the invention, the term "saponin" covers mixtures of saponins. Preferably, the mixture of saponins comprises two or more substantially pure saponins. More preferably, the two or more substantially pure saponins are from *Quillaja saponaria* in doses that are otherwise suboptimal for the individual saponins. In a particularly preferred embodiment, the combination of saponins consists essentially of

substantially pure saponins QS-7and QS-21 or, in other particularly preferred embodiments, QS-7 and QS-21-V1 or QS-7 and QS-21-V2.

Other embodiments of the invention encompasses saponins in combination with excipients. Preferably, the saponin is QS-21 and the excipients are selected from nonionic surfactants, polyvinyl pyrolidone, human serum albumin, aluminum hydroxide, agents with anesthetic action, and various unmodified and derivatized cyclodextrins. More preferably, in these embodiments, the nonionic surfactants are selected from Polysorbate 20, Polysorbate-40, Polysorbate-60, and Polysorbate-80. The polyvinyl pyrolidone may preferably be Plasdone C15, a pharmaceutical grade of polyvinyl pyrolidone. The agent having anesthetic action preferably is benzyl alcohol. Preferred cyclodextrins are hydroxypropyl-β-cyclodextrin, hydroxypropyl-γ-cyclodextrin, methyl-β-cyclodextrin.

The term "partially pure" means saponins partially separated from compounds normally associated with the saponin in its natural state.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

The present invention may also employ saponins isolated from other plant species, such as *Gypsophila* or *Saponaria officinalis*.

In one embodiment, the invention provides a method for stimulating an immune response in a subject by administering a therapeutically effective amount of saponin and oligonucleotide comprising at least one unmethylated CpG dinucleotide. The term "nucleic acid" or "oligonucleotide" refers to a polymeric form of nucleotides at least five bases in length. The nucleotides of the invention can be deoxyribonucleotides, ribonucleotides, or modified forms of either nucleotide. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased activity.

The nucleic acid molecule can include the use of phosphorothioate or phosphorodithioate rather than phosphodiesterase linkages within the backbone of the molecule, or methlyphosphorothioate terminal linkages (Kriege, et al., *Antisense and Nucl Acid Drug Dev* 6:133 (1996); Bosggs, et al., *Antisense and Nucl Acid Drug Dev*, 7:461 (1997). The phosphate backbone modification can occur at the 5′ end of the nucleic acid. The phosphate backbone modification may occur at the 3′ end of the nucleic acid, for example at the last five nucleotides of the 3′ end of the nucleic acid. Hutcherson, et al., reports in WO 95/26204 the nonsequence-specific immunostimulatory effect of phosphorothioate modified oligonucleotides. Nontraditional bases such as inosine and queosine, as well as acetyl-, thio - and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine can also be included, which are not as easily recognized by endogenous endonucleases. Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged oxygen moiety is alkylated). Nucleic acid molecules which contain a diol, such as

tetrahyleneglycol or hexaethleneglycol, at either or both termini are also included. The term "oligonucleotide" includes both single and double stranded forms of DNA.

A "CpG" or "CpG motif" refers to a nucleic acid having a cytosine followed by a guanine linked by a phosphate bond. The term "methylated CpG" refers to the methylation of the cytosine on the pyrimidine ring, usually occurring the 5-position of the pyrimidine ring. The term "unmethylated CpG" refers to the absence of methylation of the cytosine on the pyrimidine ring. Methylation, partial removal, or removal of an unmethylated CpG motif in an oligonucleotide of the invention is believed to reduce its effect. Methylation or removal of all unmethylated CpG motifs in an oligonucleotide substantially reduces its effect. The effect of methylation or removal of a CpG motif is "substantial" if the effect is similar to that of an oligonucleotide that does not contain a CpG motif. In a preferred embodiment, the CpG motif is an unmethylated CpG dinucleotide.

Preferably the CpG oligonucleotide is in the range of about 5 to 40 bases in size. For use in the instant invention, the nucleic acids can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, et al., *Tet. Let.* 22: 1859 (1981); nucleoside H-phosphonate method (Garegg, et al., *Tet. Let.* 27: 4051, (1986); Froehler, et al., *Nucl. Acid. Res.* 14:5399 (1986); Garegg, et al., *Tet. Let.* 27:4055 (1986); and Gaffney, et al., *Tet. Let.* 29:2619 (1998)). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., et al., Molecular

Coning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides. Oligonucleotides can be prepared from existing nucleic acid sequences (*e.g.*, genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endocucleases.

For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (*e.g.*, via endo-and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothiate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, *e.g.*, as described in Ts'O, et al., U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in Tullis, U.S. Patent No. 5,023,243 and Tullis, EP 092574B1) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, et al., *Chem. Rev.* 90: 544 (1990); Goodchild, *Bioconjugate Chem.* 1: 165 (1990).

For administration *in vivo*, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (*e.g.*, B-cell, monocytic cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically or covalently associated

with appropriate molecules using techniques which are well known in the art. A variety of coupling or cross-linking agents can be used, *e.g.*, protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

In preferred embodiments, the oligonucleotide containing the CpG motif may be part of a monomer or part of a multimer. Alternatively, the CpG motif may be a part of the sequence of a vector.

One embodiment of the invention covers the oligonucleotide which contains a CpG motif having the formula 5′X_1CG$X_2$3′, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

In another embodiment, the oligonucleotide sequences useful in the methods of the invention are represented by the formula:

5'N_1XCG$X_2N_2$3′

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases. In a preferred embodiment, N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length. However, nucleic acids of any size (even may kb long) can be used in the invention if CpGs are present, as larger nucleic acids are degraded into oligonucleotides inside cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' or 3' terminals and/or the consensus mitogenic

CpG motif is not a palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (*i.e.*, a sequence such as ABCDEE'D'C'B'A', in which A and A' are bases capable of forming the usual Watson-Crick base pairs.

In still another embodiment, the method of the invention includes the use of an oligonucleotide which contains a CpG motif represented by the formula:

$$5'N_1X_1X_2CGX_3X_4N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1+N_2 is from about 0-26 bases. In a preferred embodiment, N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer. CpG oligodeoxynucleotides are also preferably in the range of 8 to 30 bases in length, but may be of any size (even many kb long) if sufficient motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides of this formula do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Other CpG oligonucleotides can be assayed for efficacy using methods described herein.

In a preferred embodiment, the CpG motif comprises TCTCCCAGCGTGCGCCAT (also known as "CpG sequence 1758") or TCCATGACGTTCCTGACGTT (also known as "CpG sequence 1826") or TCGTCGTTTTGTCGTTTTGTCGTT (also known as "CpG sequence 2006").

The oligonucleotides of the invention may be chemically modified in a number of ways in order to stabilize the oligonucleotide against endogenous endonucleases. According to Davis, et al., WO 98/40100, a prolonged effect can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification (*e.g.*, a phosphorothioate or phosphorodithioate modification). For example, the oligonucleotides may contain other than phosphodiester internucleotide linkages between the 5′ end of one nucleotide and the 3′ end of another nucleotide in which the other linkage, the 5′ nucleotide phosphate, has been replaced with any number of non-traditional bases or chemical groups, such as phosphorothioate. More particularly, the phosphate backbone modification occurs at the 5′ end of the nucleic acid for example, at the first two nucleotides of the 5′ end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3′end of the nucleic acid for example, at the last five nucleotides of the 3′ end of the nucleic acid. The oligonucleotide comprising at least one unmethylated CpG dinucleotide may preferably be modified with at least one such phosphorothioate internucleotide linkages.

Oligonucleotides with phosphorothioate linkages may be prepared using methods well known in the field such as phosphoamidite (Agrawal, et al., *Proc. Natl. Acad. Sci.* 85:7079 (1988)) or H-phosphonate (Froehler, et al., *Tetrahedron Lett.* 27:5575 (1986)). Examples of other modifying chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphorothioates, phosphoroamidates, 2-O-methyls, carbamates, acetamidates, carboxymethylesters, carbonates, and phosphate triesters.

Oligonucleotides with these linkages can be prepared according to know methods (Goodchild, *Chem. Rev.* 90:543 (1990); Uhlmann, et al., *Chem. Rev.* 90:534 (1990); and Agrawal, et al., *Trends Biotechnol.* 10:152 (1992)).

In a preferred embodiment of this aspect, the inventive compositions activate the immune system. Certain preferred nucleic acids containing an unmethylated CpG have a relatively high stimulation with regard to B cell, monocyte, and/or NK cell responses. For example, as assayed by induction of cytokines, proliferative responses, lytic responses, the stimulation of the immune system may be determined.

Nucleic acids containing an unmethylated CpG can be effective in any mammal, preferably a human. Different nucleic acids containing an unmethylated CpG can cause optimal immune stimulation depending on the mammalian species. Thus, an oligonucleotide causing optimal stimulation in humans may not cause optimal stimulation in a mouse. One of skill in the art can identify the optimal oligonucleotides useful for a particular mammalian species of interest.

The term "innate immunity" as used herein refers to an immune response that is independent of a specific vaccine antigen. Cellular components involved in innate immune responses include monocytes, macrophages, natural killer cells, and polymorphonuclear cells, such as neutrophils. The term "nonspecific immunostimulator" refers to compounds that when administered to an individual or tested in vitro, increase the innate immunity of that individual or test system. Preferably, such individuals are mammals, and more preferably, the mammals are humans, however, the invention is not intended to be so limiting. Any animal which

may experience the beneficial effects of the compositions of the invention are within the scope of animals which may be treated according to the claimed invention. A nonspecific immunostimulator may enhance the immune response of the individual by increasing natural killer cell activity or cytokine production, such as interleukin-12 (IL-12) or IFNγ.

The ability of a composition to enhance innate immunity may be determined by a number of methods known to those of ordinary skill in the art. For example, the increase in natural killer cell response in mice after administration of a composition may be used as a criterion for stimulation of innate immunity. Briefly, one such method involves injecting Balb/c mice at days 1 and 2 with a test composition. Splenocytes harvested from the mice on day 3 can be tested for a natural killer cell lytic activity against a natural killer cell sensitive-cell line, such as YAC-1 cells. An additional method of determining innate immunity is to administer a test composition to a suitable species such as Balb/c mice. These mice can be challenged with an infectious agent, *e.g.*, a bacterium such as *Listeria monocytogenes* after the administration of the test compound. The ability of the test compound to stimulate the innate immune response can be tested, for example, by measuring protection against infection with the infectious agent. For example, as described herein, three days after the challenge with *Listeria*, the spleens can be removed and tested for colony forming units of *Listeria* per gram as a measure of the protective benefit of the composition.

In a first aspect of the invention, a composition comprising a saponin and an oligonucleotide comprising at least one unmethylated CpG dinucleotide may be

administered. More preferably, such a composition may increase the innate immune response in an individual or a test system to which the composition is administered. Preferably, the saponin is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin is a partially pure or substantially pure saponin from *Quillaja saponaria* Molina. Preferably, the partially pure saponin may comprise QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin is QS-21. Alternatively, the composition may comprise more than one saponin with the oligonucleotide comprising at least one unmethylated CpG dinucleotide.

In a further preferred embodiment of this first aspect, the saponin may cover a chemically modified saponin or a biologically active fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or biologically active fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2. The oligonucleotide comprising at least one unmethylated CpG dinucleotide is preferably a monomer or multimer. Another preferred embodiment of the CpG motif is as a part of the sequence of a vector.

Yet another embodiment of this first aspect is directed to the oligonucleotide comprising at least one unmethylated CpG dinucleotide, wherein the oligonucleotide is modified. The particular modification may comprise at least one phosphorothioate internucleotide linkage. Further, the oligonucleotide having at least one unmethylated CpG dinucleotide may comprise a CpG motif having the formula $5'X_1CGX_2 3'$, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine,

or thymine, and X_2 is cytosine, thymine, or adenine. The CpG motif may preferentially be TCTCCCAGCGTGCGCCAT or TCCATGACGTTCCTGACGTT, or TCGTCGTTTTGTCGTTTTGTCGTT .

The term "composition" herein refers to a composition capable of stimulating an innate immune response. A composition, according to the invention, would produce innate immunity against disease in individuals. A composition comprising a saponin and an oligonucleotide comprising at least one unmethylated CpG dinucleotide of the present invention may be administered to an individual to enhance the immune response prior to or after exposure to a pathogen or tumor. Preferably, the composition stimulates innate immunity. More preferably, the composition enhances a protective natural killer cell response.

The composition of the invention comprising both saponin and CpG-containing oligonucleotide may enhance the immune response, *e.g.*, the innate immune response, in a positive synergistic manner. In one embodiment, the innate immune response is natural killer cell response. The term "positive synergistic effect" and "positive synergistic manner" mean the enhancement by the inventive composition, *e.g.*, a saponin plus a CpG-containing oligonucleotide, on immune response to a level that is greater than the addition of the response to the components used individually. The synergistic effect of the composition of oligonucleotide plus saponin described herein may be shown as an increase in the maximum expected immune response, *e.g*, the NK cell response, over the addition of the response caused by the oligonucleotide alone and the response caused by the saponin alone.

In a second aspect, the invention is directed to a method for increasing the innate immune response in an individual or a test system comprising administering an effective amount of a composition comprising a saponin with or without an oligonucleotide comprising at least one unmethylated CpG dinucleotide. Preferably, the saponin is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin is a partially pure or a substantially pure saponin from *Quillaja saponaria* Molina. The method may also embody a composition comprising more than one substantially pure saponin and an oligonucleotide comprising at least one unmethylated CpG dinucleotide. The substantially pure saponin is preferably QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin is QS-21. In a further preferred embodiment, the saponin may cover a chemically modified saponin or a biologically active fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract. In a preferred embodiment of the method, the oligonucleotide containing at least one CpG motif is preferably a monomer or a multimer. Another preferred embodiment of the method includes the CpG motif as a part of the sequence of a vector. Yet another embodiment is directed to the method wherein the oligonucleotide comprises at least one unmethylated CpG dinucleotide, and wherein furthermore the oligonucleotide may be chemically modified to stabilize the oligonucleotide against endogenous endonucleases. The modification may comprise at least one phosphorothioate internucleotide linkage. Further, the method may be directed, in part, to the oligonucleotide having at least one unmethylated CpG dinucleotide comprising a CpG motif having the formula $5'X_1CGX_2 3'$, wherein at least one nucleotide separates

consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred method, the unmethylated CpG motif is TCTCCCAGCGTGCGCCAT, TCCATGACGTTCCTGACGTT, or TCGTCGTTTTGTCGTTTTGTCGTT.

A third aspect of the invention provides for methods for stimulating innate immunity comprising administering an effective amount of a composition comprising a saponin to an individual. Preferably, the method provides that the saponin is derived from *Quillaja saponaria*, and more preferably, the saponin is chemically modified or comprises a substantially pure saponin. In a preferred embodiment of the third aspect, the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21, and more preferably, the substantially pure saponin comprises QS-21. The method, according to the third aspect of the invention, preferably further increases an innate immune response when administered to a mammal or a human. Still another preferred embodiment is directed to the method for further enhancing a natural killer cell response.

Further, numerous infectious diseases and cancers are suitable for prevention or treatment by the enhanced innate immune response. Viral diseases that can be treated or prevented by the methods of the present invention include, but are not limited to, those caused by hepatitis type A, hepatitis type B, hepatitis type C, feline leukemia virus, feline immunodeficiency virus, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, vhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus,

echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), human immunodeficiency virus type II (HIV-II), rabies virus, and hoof and mouth virus.

Bacterial diseases than can be treated or prevented by methods of the present inventions are caused by bacteria including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria, legionella, Yersinia, *Helobacter pylori*, *Staphylococcus aureus*, anthrax, diphtheria, *Escherichia coli*, Lyme disease, *Listeria monocytogenes*, pneumococcus, *Francisella tularensis*, *Salmonella*, or tuberculosis.

Protozoal diseases that can be treated or prevented by the methods of the present invention are caused by protozoa including, but not limited to, leishmania, kokzidioa, trypanosoma, Plasmodium and *Babeosis bovis*.

Parasitic diseases that can be treated or prevented by the methods of the present invention are caused by parasites including, but not limited to, chlamydia and rickettsia. Other pathogens not listed above may be suitable for treatment by the enhanced innate immune response. In addition, cancers may be suitable for treatment by the enhanced innate immune response. Cancers that can be treated or prevented by the methods of the present invention include, but not limited to, human sarcomas and carcinomas, e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal

cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non Hodgkin's disease), multiple myeloma, Waldenstrobm's macroglobulinemia, and heavy chain disease.

In a specific embodiment the cancer is metastatic. In another specific embodiment, the patient having a cancer is immunosuppressed by reason of having undergone anticancer therapy (*e.g.*, chemotherapy radiation) prior to administration of the compositions of the invention. In another specific embodiment, the cancer is a tumor.

The saponins and oligonucleotides comprising at least one unmethylated CpG dinucleotide (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise a saponin and an oligonucleotide comprising at least

one unmethylated CpG dinucleotide and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agent, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrocholoric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or

plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringbility exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier also can be a solvent or dispersion medium containing, for example, sterile water, salt solutions (such as Ringer's solution or saline), alcohols, gelatin, talc, viscous paraffin, fatty acid asters, hydroxymethylcellulose, polyvinyl pyrolidone, calcium carbonate, carbohydrates such as lactose, sucrose, dextrose, mannose, albumin, starch, cellulose, silica gel, polyethylene glycol (PEG), dried skim milk, rice flour, magnesium stearate, and the like, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, paragens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be

brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a

lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.* with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polyactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein, et al., U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitation inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from assays and animal models described herein. Such information can be used to more accurately determine useful doses in humans.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including, but not limited to, the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment, or preferably, can include a series of treatments. The initial dose may be followed up with a booster dosage after a period of about 2 days to 2 weeks to maintain the innate immunity. Further booster dosages may also be administered.

The effective compositions of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert acceptable carrier may preferably be used or any such acceptable carrier in which the compositions of the present invention have suitable solubility properties for use of the present invention.

Methods of administration will vary in accordance with the type of disorder and

disease sought to be controlled or eradicated. The dosage of the composition will be dependent on a number of factors, including the route of administration. A person of ordinary skill in the art may easily and readily titrate the dosage for an enhanced immune response.

The actual effective amounts of compounds can vary according to the specific composition being utilized, the mode of administration, and the age, weight, and condition of the individual. As used herein, an effective amount of the drug is an amount which elicits or boosts an innate immune response. Dosages for a particular individual may be determined by a person of ordinary skill in the art using conventional considerations, *e.g.*, by a means of appropriate, conventional pharmacological protocol.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the compositions in a pharmaceutically acceptable form. The composition in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the composition may be lyophilized or desiccated; in the instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the composition to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or

syringe, preferably packaged in sterile form, for injecting the composition, and/or a packaged alcohol pad. Instructions are optionally included for administration of the composition by a clinician or by a patient.

Various cytokines, antibiotics, and other bioactive agents also may be co-administered with the compositions described herein. For example, various known cytokines, *i.e.*, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7, interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), IL-12, interferon-α (INFα), interferon-β (INFβ), interferon-γ (INFγ), tumor necrosis factor a, tumor necrosis factor β (TNFβ), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF-β) may be co-administered with the composition in order to maximize the physiological response. However, it is anticipated that other but as yet undiscovered cytokines may be effective in the invention. In addition, conventional antibiotics may be co-administered wit the compositions. The choice of suitable antibiotics will, however, be dependent upon the disease in question.

The following examples are meant to be illustrative and not limiting in any way.

EXAMPLES

A well-established animal model was used to assess whether different formulations of CpG oligodeoxynucleotide and QS-21 together or alone could function

as stimulators of innate immunity. In brief, experiments were set up to compare QS-21 to a recently reported immunostimulatory CpG motif. An immunostimulatory CpG sequence (*e.g.*, 1826), reported to serve as an adjuvant in mice, was selected. One experiment evaluated whether the CpG motif alone, QS-21 alone, or the CpG/QS-21 combination may serve to increase innate immunity by activation of natural killer cells.

The experiments were performed using materials from the following suppliers: QS-21 and QS-7 (Aquila Biopharmaceuticals); CpG oligodeoxynucleotides included the phosphorothiate-modified sequences 1826 TCCATGACGTTCCTGACGTT and 2006 TCGTCGTTTTGTCGTTTTGTCGTT (Life Technologies (Gibco)), murine recombinant IL-12 (Pharmingen), andYAC-1 cells (ATCC), a natural killer cell-sensitive target line.

Example 1

Natural Killer Cell Activity Induced by QS-21 and CpG/QS-21

Assessment of natural killer cell activity was carried out by an adaptation of a published method (Hashimoto et al., *J. Immunol.* 163: 583 (1999)). Balb/c mice (4 per group, female, 8-10 weeks of age) were administered one of five different candidate compositions at days 1 and 2. The compositions evaluated were (1) saline (negative control), (2) 10 ug QS-21, (3) 10 ug CpG (sequence 1826), (4) 0.5 ug murine IL-12 (positive control for NK cell activation), and (5) a combination of 10 ug QS-21 and 10 ug CpG in 0.2 ml saline. All test compositions were administered subcutaneously except for murine IL-12, which was administered intraperitoneally. Splenocytes were removed from the mice at day 3 for use as effector cells in the natural killer cell assay. Such cells were immediately used in a standard ^{51}Cr release lysis assay. YAC-1 cells

(loaded with ^{51}Cr) were used as target cells. The lysis of this NK cell-sensitive line is indicative of NK cell activation in the splenocyte population.

The results, as shown in the graphic representation of Figure 1, indicate that minimal lysis (less than 20% at 100:1 effector to target ratio) was observed after the administration of saline. CpG alone slightly enhanced the NK cell activity. Surprisingly, QS-21 alone induced an NK cell response that was higher than CpG and that was nearly equivalent to the positive control, murine IL-12. Still more surprisingly, the combination of QS-21 and CpG induced the strongest NK cell response.

Example 2
Time Dependence of Natural Killer Cell Activity Induced by QS-21 and CpG/QS-21

The time dependence of the administration of CpG/QS-21 on natural killer cell activation was investigated. Balb/c mice (5 per group, female, 8-10 weeks of age) were administered a mixture of 10 ug QS-21 and 10 ug CpG sequence 1826 in a total volume of 0.2 ml by subcutaneous route seven days before (-7 d), three days before (-3 d), two days before (-2 d), and one day before (-1 d) assay of natural killer cell activity. Splenocytes were removed from the mice at day 0 for use as effector cells in the natural killer cell assay. YAC-1 cells (loaded with ^{51}Cr) were used as target cells. Natural killer cell lysis was apparent if the formulation of QS-21/CpG was administered one, two, or three days prior to the assay, but not if the formulation was administered seven days prior to the assay (Figure 2). This confirms the transient nature of the natural killer cell activity.

Example 3

Dose Response of QS-21, QS-7, and CpG Sequence 1826

Balb/c mice (5 per group, female, 8-10 weeks of age) were administered individually QS-7, QS-21, or CpG sequence 1826 at three different dose levels (3, 10, 30 ug) to determine a dose response curve for these individual compounds. The compositions evaluated were (1) saline (negative control), (2) 3 ug QS-21, (3) 10 ug QS-21, (4) 30 ug QS-21, (5) 3 ug QS-7, (6) 10 ug QS-7, (7) 30 ug QS-7, (8) 3 ug sequence CpG 1826, (9) 10 ug CpG sequence 1826, and (10) 30 ug CpG sequence 1826. All test compositions were administered subcutaneously at day 1 and day 2. Splenocytes were removed from the mice at day 3 for use as effector cells in the natural killer cell assay. YAC-1 cells (loaded with ^{51}Cr) were used as target cells.

The results, as shown in the graphic representation of Figure 3, confirm that QS-21, QS-7, and CpG sequence 1826 enhance NK activity in a dose dependent fashion. The NK cell activity induced by QS-21 or CpG sequence 1826 was higher than that induced by QS-7 at an equivalent dose. This experiment confirmed that NK activity could be induced by another saponin.

Example 4

NK Activity Induced by QS-21 and/or QS-7 and CpGs Sequences 1826 and 2006

This experiment evaluated the natural killer cell stimulating activity induced by various formulations: (1) QS-21 (10 ug), (2) QS-7 (10 ug), (3) CpG sequence 1826 (10 ug), (4) CpG sequence 2006 (10 ug), (5) QS-21 (10 ug) + CpG sequence 1826 (10 ug), (6) QS-21 (10 ug) + CpG 2006 (10 ug), (7) QS-7 (10 ug) + CpG sequence 1826 (10 ug), (8) QS-7 (10

ug) + CpG sequence 2006 (10 ug), (9) QS-7 (10 ug) and QS-21 (10 ug) and (10) saline. Balb/c mice (5 per group, female, 8-10 weeks of age) were administered the above formulations by subcutaneous route on day 1 and day 2. Splenocytes were removed from the mice at day 3 for use as effector cells in the natural killer cell assay. YAC-1 cells (loaded with ^{51}Cr) were used as target cells.

As evident in the graphic representation of Figure 4, the results show that the three formulations inducing the strongest response are QS-21/CpG sequence 1826, QS-21/CpG sequence 2006, and QS-7/CpG sequence 1826. This indicates that mixtures of alternate CpG (sequence 2006) with QS-21 also lead to a heightened NK cell response; likewise mixtures of alternate saponins (QS-7) with CpG can also lead to a heightened NK cell response.

Example 5
Protection of Mice from *Listeria Monocytogenes* by Administration of Formulations that Enhance Innate Immunity

Another method of demonstration of enhanced innate immunity is in an *in vivo* challenge model. The protective benefit of formulations of QS-21 or QS-21/CpG was demonstrated in a *Listeria monocytogenes* challenge model in Balb/c mice. Immunity to *Listeria monocytogenes* can be mediated by innate immunity and is believed to rely on cytokines produced by natural killer cells (Harty, et al., *Curr. Opin. Immunol* 8:526) (1996)). Hence, this challenge model was used as a demonstration of the benefit of enhanced innate immunity raised by administration of the inventive compositions.

Balb/c mice (5 per group, female, 8-10 weeks of age) were administered the following formulations on day 0: Group 1: saline, subcutaneous route. Group 2: 10 ug QS-21 and 10 ug of CpG sequence 1826, subcutaneous route. Group 3: 10 ug QS-21, subcutaneous route. Group 4: 10 ug CpG sequence 1826, subcutaneous route. Group 5: 0.5 ug recombinant murine IL-12, intraperitoneal route. A total volume of 0.2 ml was administered. On day 3, mice were challenged by the intraperitoneal route with 10^5 colonies of *Listeria monocytogenes* strain 10403s. Spleens were removed at 96 hours after challenge, homogenized, and then cultured in serial 10-fold dilutions overnight on agar plates. *Listeria monocytogenes* colonies were counted, the number of organisms per spleen determined, and then the geometric mean and standard error were determined for each group. A two-tailed, paired student's t-test of the log10 colonies/spleen was used to show statistical significance.

Figure 5 is a graphic representation showing the results of the challenge. The group with the highest spleen colony count was the group receiving saline (control group). All other groups had lower mean colony counts in spleen. The lowest colony counts were in the CpG + QS-21 group and in the QS-21 group, both of which reached statistical significance ($p<0.05$). This suggests that these two formulations raise an innate immunity that is protective against a challenge with a bacterium.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

We claim:

1. A composition comprising:

(a) a saponin; and

(b) an oligonucleotide comprising at least one unmethylated CpG dinucleotide.

2. The composition as claimed in claim 1, wherein the saponin is derived from *Quillaja saponaria.*

3. The composition as claimed in claim 2, wherein the saponin is chemically modified.

4. The composition as claimed in claim 2, wherein the saponin comprises a substantially pure saponin.

5. The composition as claimed in claim 4, wherein the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21.

6. The composition as claimed in claim 5, wherein the substantially pure saponin comprises QS-21.

7. The composition as claimed in claim 1, wherein the oligonucleotide is chemically modified.

8. The composition as claimed in claim 7, wherein the oligonucleotide is modified with at least one phosphorothioate internucleotide linkage.

9. The composition as claimed in claim 1, wherein the oligonucleotide comprises a CpG motif having the formula $5'X_1CGX_2 3'$, wherein at least one nucleotide

separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

10. The composition as claimed in claim 9, wherein the CpG motif comprises TCCATGACGTTCCTGACGTT or TCGTCGTTTTGTCGTTTTGTCGTT.

11. The composition as claimed in claim 1, wherein the composition increases an innate immune response when administered to a mammal.

12. The composition as claimed in claim 1, wherein the composition increases an innate immune response when administered to a human.

13. The composition as claimed in claim 1, wherein the composition increases an innate immune response when administered to a mammal other than a human.

14. The composition as claimed in claim 11, wherein the composition further enhances a natural killer cell response.

15. The composition as claimed in claim 14, wherein the composition further enhances a natural killer cell response in a positive synergistic manner.

16. A method for stimulating innate immunity comprising administering an effective amount of a composition comprising:

(a) a saponin; and

(b) an oligonucleotide comprising at least one unmethylated CpG motif to an individual.

17. The method as claimed in claim 16, wherein the saponin is derived from *Quillaja saponaria*.

18. The method as claimed in claim 16, wherein the saponin is chemically modified.

19. The method as claimed in claim 17, wherein the saponin comprises a substantially pure saponin.

20. The method as claimed in claim 19, wherein the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21.

21. The method as claimed in claim 20, wherein the substantially pure saponin comprises QS-21.

22. The method as claimed in claim 16, wherein the oligonucleotide is chemically modified.

23. The method as claimed in claim 22, wherein the oligonucleotide is modified with at least one phosphorothioate internucleotide linkage.

24. The method as claimed in claim 16, wherein the oligonucleotide comprises a CpG motif having the formula $5'X_1CGX_2 3'$, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

25. The method as claimed in claim 24, wherein the CpG motif comprises TCCATGACGTTCCTGACGTT or TCGTCGTTTTGTCGTTTTGTCGTT.

26. The method as claimed in claim 16, wherein the composition stimulates an innate immune response when administered to a mammal.

27. The method as claimed in claim 16, wherein the composition stimulates an innate immune response when administered to a human.

28. The method as claimed in claim 16, wherein the composition stimulates an innate immune response when administered to a mammal other than a human.

29. The method as claimed in claim 16, wherein the method further enhances a natural killer cell response.

30. The method as claimed in claim 16, wherein the method further enhances a natural killer cell response in a positive synergistic manner.

31. A method for stimulating innate immunity comprising administering an effective amount of a composition comprising a saponin to an individual.

32. The method as claimed in claim 31, wherein the saponin is derived from *Quillaja saponaria.*

33. The method as claimed in claim 32, wherein the saponin is modified.

34. The method as claimed in claim 32, wherein the saponin comprises a substantially pure saponin.

35. The method as claimed in claim 34, wherein the substantially pure saponin comprises QS-7, QS-17, QS-18, or QS-21.

36. The method as claimed in claim 35, wherein the substantially pure saponin comprises QS-21.

37. The method as claimed in claim 32, wherein the composition stimulates an innate immune response when administered to a mammal.

38. The method as claimed in claim 32, wherein the composition stimulates an innate immune response when administered to a human.

39. The method as claimed in claim 32, wherein the composition stimulates an innate immune response when administered to a mammal other than a human.

40. The method as claimed in claim 32, wherein the method further enhances a natural killer cell response.

41. The method as claimed in claim 40, wherein the method further enhances a natural killer cell response in a positive synergistic manner.

42. The composition as claimed in claim 12, wherein the composition further enhances a natural killer cell response.

43. The composition as claimed in claim 13, wherein the composition further enhances a natural killer cell response.

ABSTRACT

Compositions comprising oligonucleotides comprising at least one unmethylated CpG dinucleotide and saponin and the use thereof for stimulating innate immunity and enhancing natural killer cell activity are disclosed.

Figure 1

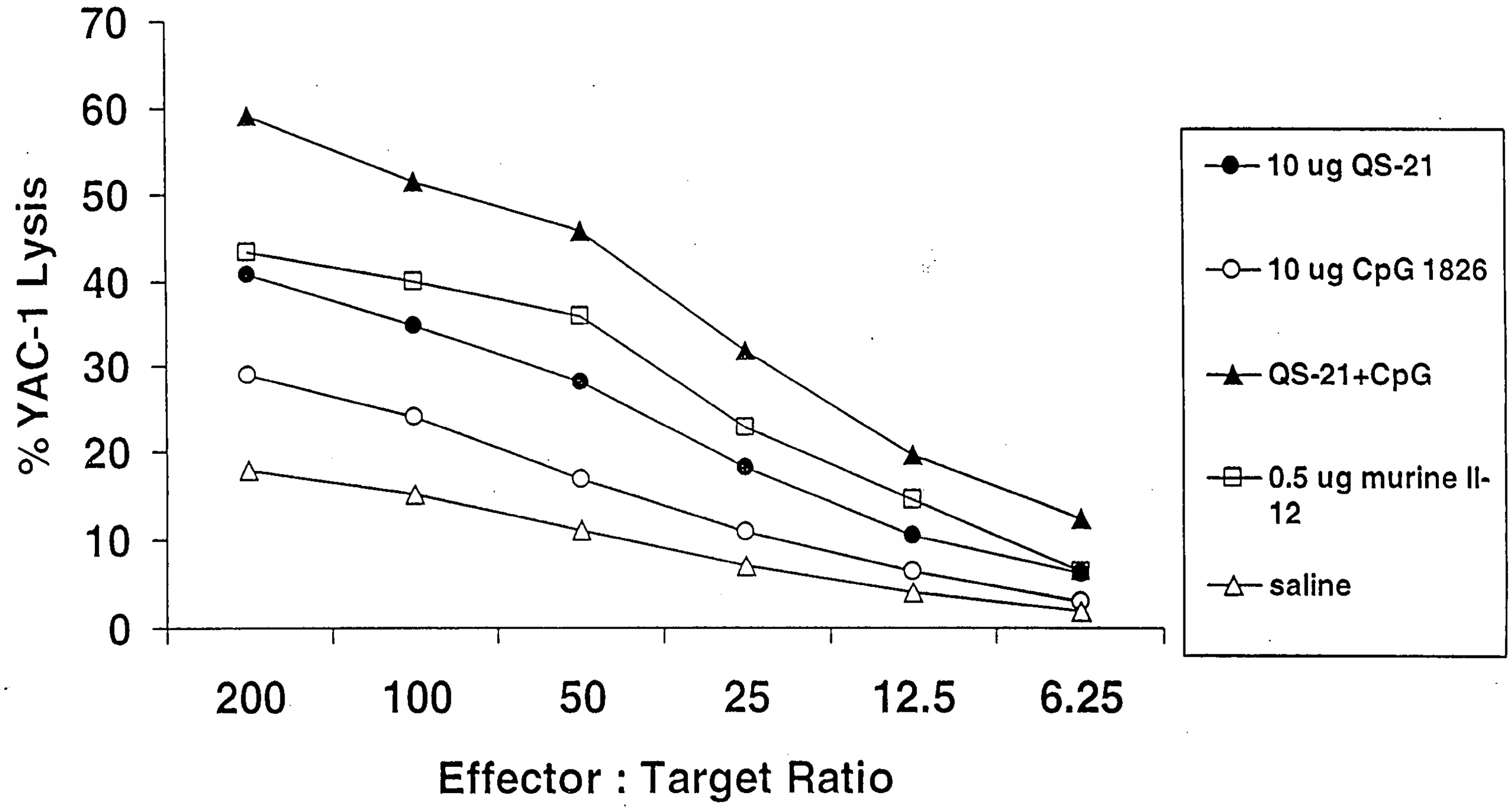

% YAC-1 Lysis
70
60
50
40
30
20
10
0
200
100
50
25
12.5
6.25
Effector : Target Ratio
10 ug QS-21
10 ug CpG 1826
QS-21+CpG
0.5 ug murine Il-12
saline

Figure 2

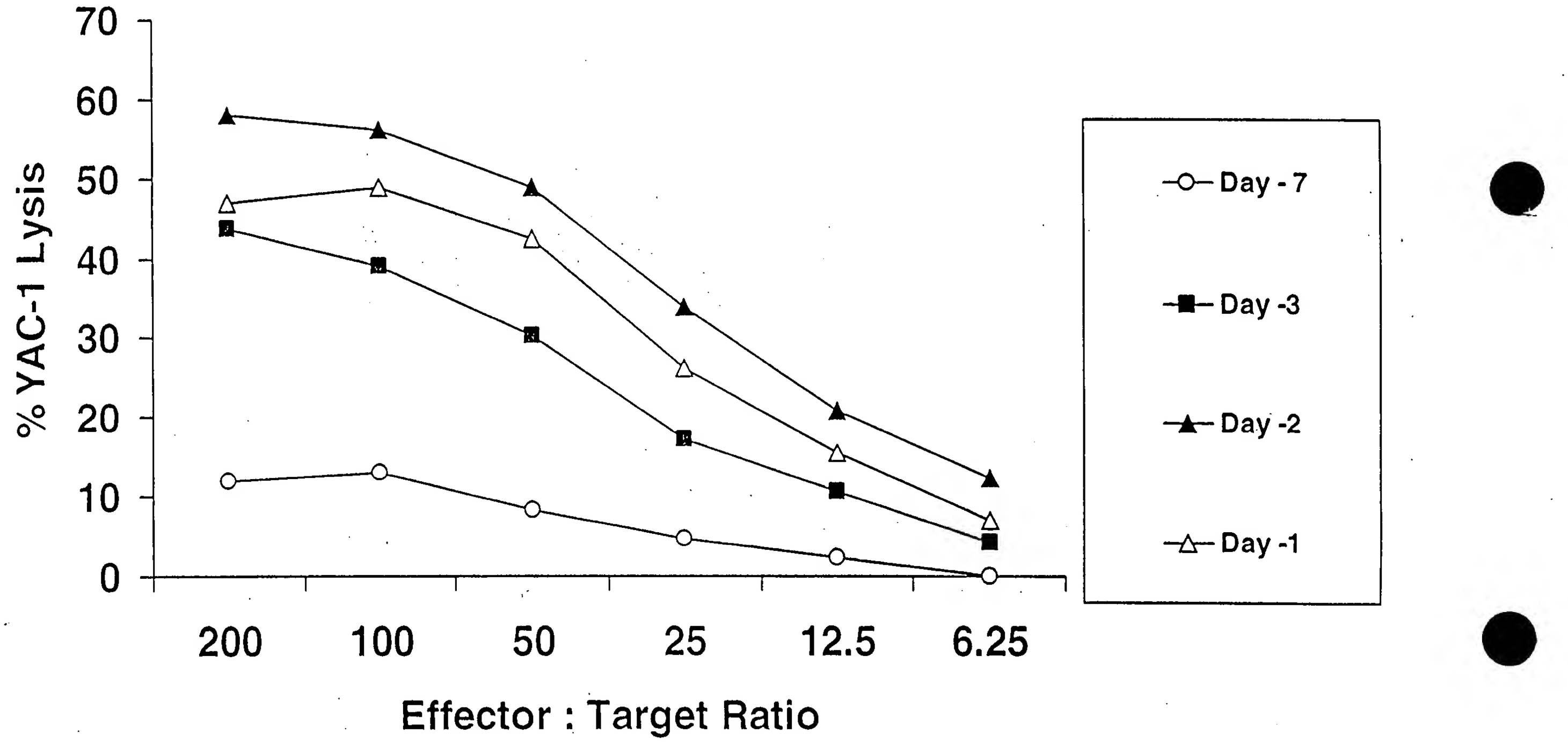

70
60
50
40
30
20
10
0
% YAC-1 Lysis
200
100
50
25
12.5
6.25
Effector : Target Ratio
Day - 7
Day -3
Day -2
Day -1

Figure 3

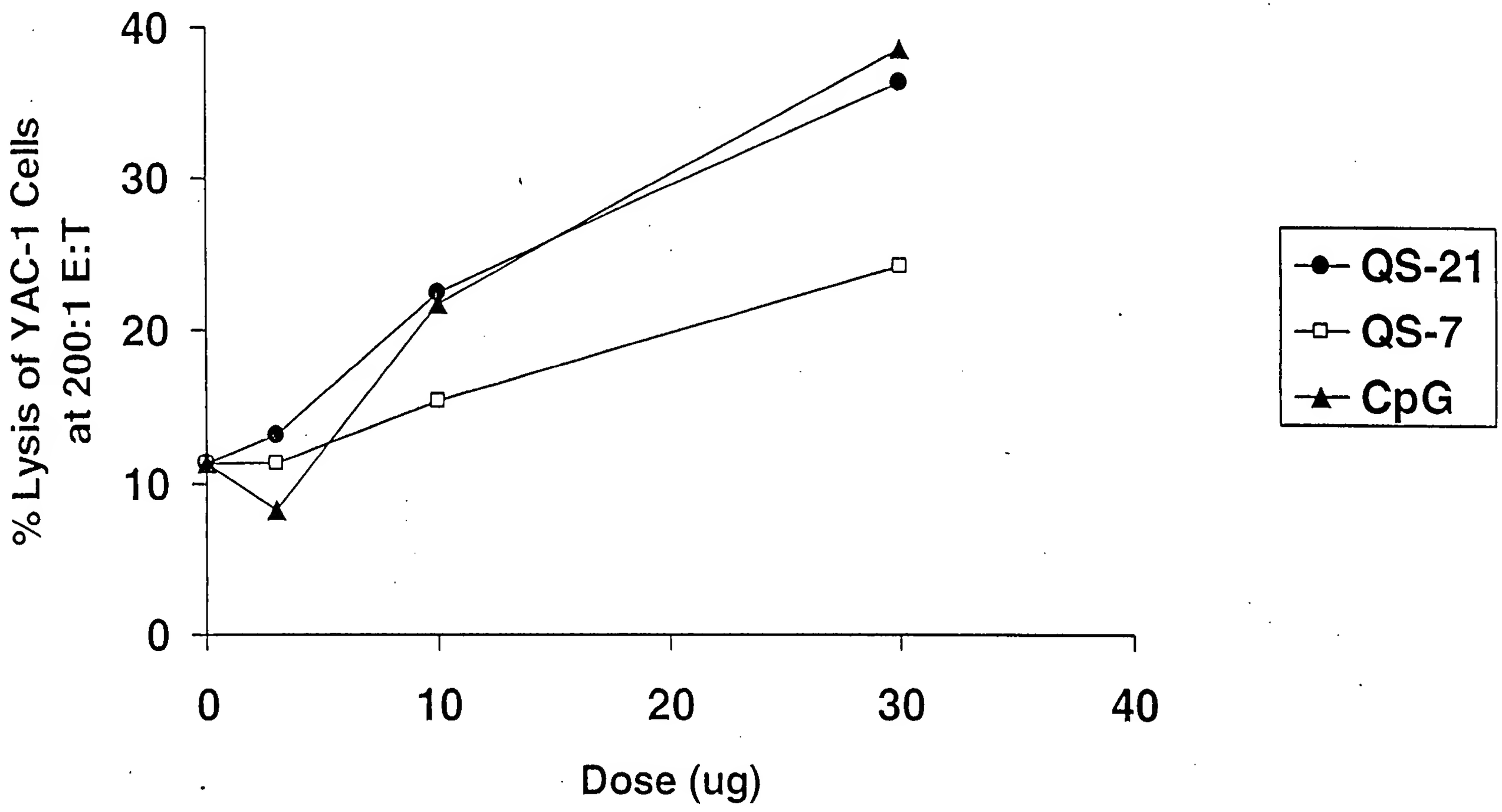
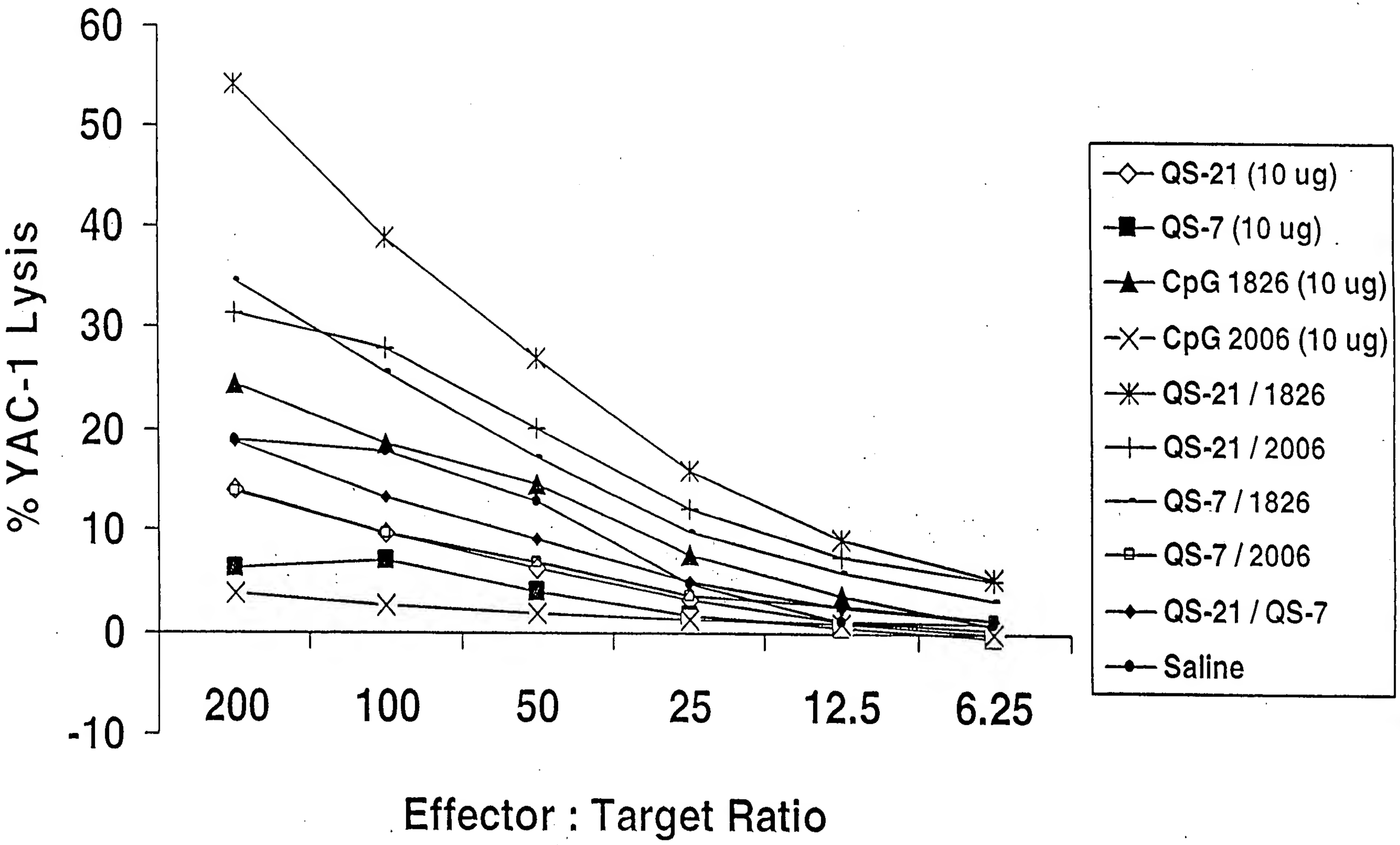

% Lysis of YAC-1 Cells
at 200:1 E:T
40
30
20
10
0
0
10
20
30
40
Dose (ug)
QS-21
QS-7
CpG

Figure 4


60
50
40
30
20
10
0
-10
% YAC-1 Lysis
200
100
50
25
12.5
6.25
Effector : Target Ratio
QS-21 (10 ug)
QS-7 (10 ug)
CpG 1826 (10 ug)
CpG 2006 (10 ug)
QS-21 / 1826
QS-21 / 2006
QS-7 / 1826
QS-7 / 2006
QS-21 / QS-7
Saline

APPENDIX B
PENDING CLAIMS AS OF APRIL 23, 2003
U.S. PATENT APPLICATION SERIAL NO. 09/760,506
ATTORNEY DOCKET NO. 8449-153-999

31. (amended) A method of treating cancer comprising administering to an individual in need thereof an effective amount of a composition comprising a *Quillaja saponaria* saponin, wherein said effective amount stimulates innate immunity.

33. (amended) The method as claimed in claim 31, wherein said *Quillaja saponaria* saponin is chemically modified.

34. (amended) The method as claimed in claim 31, wherein said *Quillaja saponaria* saponin is substantially pure.

35. (amended) The method as claimed in claim 34, wherein said substantially pure *Quillaja saponaria* saponin is QS-7, QS-17, QS-18, or QS-21.

36. (amended) The method as claimed in claim 35, wherein said substantially pure *Quillaja saponaria* saponin is QS-21.

37. (amended) The method as claimed in claim 31, wherein said individual is a mammal.

38. (amended) The method as claimed in claim 31, wherein said individual is a human.

40. (amended) The method as claimed in claim 31, wherein said effective amount of a composition comprising a *Quillaja saponaria* saponin is an amount sufficient to enhances a natural killer cell response.

44. (new) The method as claimed in claim 40, wherein said enhanced natural killer cell response is evaluated by an *in vitro* assay comprising:

(a) contacting natural killer cell-sensitive target cells with effector cells, wherein said effector cells are from said individual that has been administered said composition;

(b) determining the level of lysis of said natural killer cell-sensitive target cells;

wherein increased lysis of natural killer cell-sensitive target cells contacted with effector cells from said individual that has been administered said composition as compared to natural killer cell-sensitive target cells contacted with effector cells from an individual that has not been administered said composition indicates an enhanced natural killer cell response.

AIDS RESEARCH REVIEWS

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Novel Adjuvants from *Quillaja saponaria* Molina

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INTRODUCTION

Modulation of immune responses to HIV-1 vaccines, particularly subunit vaccines, may require the development of new adjuvants. One such adjuvant can be found in a unique group of compounds, the plant saponins. In particular, certain saponins from *Quillaja saponaria* Molina are potent stimulators of immune responses. The biological activities of crude and partially purified extracts from *Quillaja saponaria* have been described in numerous published reports. Unfortunately, due to lack of purification, it was not clear which components of the extracts were responsible for these biological activities. We have purified the major adjuvant active components from *Q. saponaria*, allowing concurrent structure and function characterization. In this chapter, we review the immunological and structural characterization of this important new adjuvant class, with particular focus on QS-21, the best characterized of the *Q. saponaria* adjuvants.

Quillaja saponaria Molina is a species of tree indigenous to South America. The bark of this tree is rich in triterpene glycoside saponins, representing up to 10% of the weight of the bark. The bark extracts have numerous industrial uses for which surface active agents are needed. As such, the bark is an export product of several countries, such as Chile. The extracts of Q. *saponaria* have also been shown to contain components useful in vaccine applications.

Extracts of the bark of *Quillaja saponaria* were first shown to enhance the protective effect of a foot-and-mouth-disease vaccine in cattle (1). However, these crude saponin extracts consisted predominantly of tannins in addition to the saponin fraction. In 1974, Dalsgaard (2) isolated the saponin fraction away from the contaminating tannins and showed that all of the adjuvant activity was contained in the saponin fraction. He further purified this fraction by a combination of gel filtration and ion-exchange chromatography to produce a fraction that is now known at Quil A. Quil A has been utilized extensively as an immunological adjuvant for T-dependent antigens (2,3). It is also a critical component of immunestimulating complexes (ISCOMs) (4).

Quil A was shown to be a very heterogeneous mixture (5), consisting of at least 20 peaks when analyzed by reverse-phase high-performance liquid chromatography (HPLC). Attempts to purify these components to homogeneity in aqueous systems were ineffective, although partial resolution of adjuvant activity away from irritating substances was achieved by gel-filtration chromatography (6). The difficulty in purification was due to the detergent nature of the saponin fraction; mixed micelle formation between the different saponins and other lipophilic contaminants prevented effective separation in aqueous solution.

We have recently identified the adjuvant active components from Q. *saponaria* (7). In our study, the total saponin fraction from Q. *saponaria* was resolved into individual, distinct saponins by disruption of the micellar interactions in organic solvent and subsequent purification by HPLC. These purified components were evaluated for adjuvant activity in mice using bovine serum albumin (BSA) as the immunogen and by measuring increases in antibody titers. The four predominant peaks in the saponin fraction were identified as adjuvant active components; these peaks were designated QS-7, QS-17, QS-18, and QS-21. The purification of these distinct saponins has allowed us to initiate studies to characterize the purified saponins both immunologically and chemically.

STIMULATION OF ANTIBODY RESPONSES

Increases in antigen-specific IgG titers in mice in response to formulations containing QS-21 as adjuvant have been observed with the antigens BSA (7), cytochrome b_5 (7), and ovalbumin (OVA) (8). The antibody titers induced in mice by two intradermal immunizations with 20 μg of purified saponin with either the antigen cytochrome b_5 (7) or OVA (data not shown) were found to be comparable to the titers

induced by Freund's complete adjuvant and were higher than the titers induced by aluminum hydroxide.

The dose effect of QS-7, QS-17, QS-18, and QS-21 on the stimulation of antigen-specific IgG was assessed in both CD-1 and C57BL/6 mice. Two immunizations of formulations containing BSA or OVA and varying doses of these purified saponins (over a dose range up to 40 μg with QS-18 and up to 80 μg with QS-7, 17, and 21) were given to group, of five mice (7–9). The minimum optimal dose was determined to be approximately 5 μg each of these saponins; the antibody titers reached a maximum with purified saponin doses of 5 μg and higher.

IgG SUBCLASS SWITCHING

Adjuvants are known to regulate IgG subclasses (10), a factor that is an important component of an adjuvant's potential protective role against viral and bacterial infections. Mouse IgG_2 subclasses fix complement at a higher level than IgG_1 (11). Additionally, IgG_{2a} and IgG_{2b} bind strongly to Fc receptor, a response critical to antibody-dependent cellular cytotoxicity (12). Hence, the ability of an adjuvant to change an antibody response from predominantly an IgG_1 response to a broadened response containing IgG_1, IgG_{2b}, and IgG_{2a} may be an essential component of protective responses to pathogens.

The purified saponins clearly induce subclass switching. Immunization of CD-1 mice with the antigen cytochrome b_5 and QS-7, QS-17, QS-18, or QS-21 broadens the antibody response to include high levels of IgG_{2a} and IgG_{2b} isotypes compared to immunization with antigen alone (7). An example of the isotype switching with another T-dependent antigen, OVA, is shown in Table 1. OVA-specific IgG_1, IgG_{2b}, and IgG_{2a} are significantly increased after three intradermal immunizations of C57BL/6 mice with OVA and QS-21 compared to immunization with OVA alone. The IgG subclass switching has also been demonstrated with BSA/QS-21 and with recombinant FeLV gp70/QS-21 and HIV-1 gp160/QS-21 vaccines (data not shown). The induction of IgG_{2a} suggests the possible activation of Th_1 cells and subsequent

Table 1 Influence of QS-21 on Antibody Titers to T-Dependent Antigen

Formulation[a]	IgG_1	IgG_{2b}	IgG_{2a}
OVA (25 μg)	140	<10	<10
OVA (25 μg)	9100	2000	2400
QS-21 (10 μg)			

[a]C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10, and 12 weeks of age with the indicated formulations. Sera were collected 2 weeks after the last immunization and were analyzed for antibody to ovalbumin by EIA.

production of the cytokine interferon-γ as part of the cascade induced by the saponin adjuvants; this cascade has been proposed by Karagouni et al. (10), who also noted a stimulation of IgG_{2a} production in response to immunization with crude saponin.

LACK OF INDUCTION OF IgE

Some crude saponin preparations have induced allergic responses mediated by the production of IgE (13). However, the purified saponins QS-7, -17, -18, and -21 did not induce IgE with the antigen cytochrome b_5 (7), suggesting that a nonsaponin component(s) of the crude preparations was responsible for stimulation of IgE titers. The low levels of IgE measured experimentally are consistent with the high levels of IgG_{2a} elicited by these purified saponin adjuvants. Typically, induction of IL-4 [shown to be correlated with production of IgE antibody in mice (14)] is inversely correlated with induction of interferon-γ, which is associated with the production of IgG_{2a} (15).

INDUCTION OF IMMUNOLOGICAL MEMORY

Also critical to protective immune response is the induction of immunological memory. Not all adjuvants invoke memory responses. Studies with recombinant HIV-1 glycoproteins in BALB/c mice indicate that the saponin QS-21 is effective in inducing anamnestic responses. BALB/c mice immunized twice with 10-μg doses of recombinant HIV-1 gp160/aluminum hydroxide/10 μg of QS-21 responded with significant increases in antibody titers 1 week after challenge with inactivated HIV-1 IIIB, whereas mice immunized with the same formulation without QS-21 did not respond (16). The kinetics of the rapid titer increase are consistent with a memory response. Memory responses were also observed to an FeLV vaccine containing recombinant FeLV gp70, aluminum hydroxide, and QS-21. Specific pathogen-free cats immunized twice with this vaccine developed a rapid neutralizing response in response to intraperitoneal challenge with infectious FeLV (17).

QS-21 AS ADJUVANT FOR T-INDEPENDENT ANTIGENS

Flebbe and Braley-Mullen (3) demonstrated that Quil-A is an effective adjuvant for a hapten coupled to the T-independent antigens Ficoll, lipopolysaccharide (LPS), and *Brucella abortus*. Because of the heterogeneity of Quil A, it was not clear which component(s) was responsible for this activity. At least one purified saponin, QS-21, has been shown to significantly augment antibody response to the T-independent antigen *E. coli* 055:B5 polysaccharide (O-PS) (18), prepared by acid hydrolysis of phenol-extracted LPS. This response was not due to residual native LPS activity, because similar results were observed in the LPS nonresponder mouse strain C3H/HeJ. The antibody responses raised to native LPS after two intradermal immunizations with

Table 2 Influence of QS-21 on Antibody Titers to T-Independent Antigen

Formulation[a]	IgG_1	IgG_{2b}	IgG_{2a}
O-PS (100 μg)	15.000	3900	9800
O-PS (100 μg) QS-21 (15 μg)	82.000	99.000	115.000

[a]C3H/HeJ mice (19 per group) were immunized intradermally at 8 and 10 weeks of age with the indicated formulations. Sera were collected 1 week after the last immunization and were analyzed for antibody to native *E. coli* lipopolysaccharide by EIA.

O-PS and 15 μg of QS-21 are described in Table 2. Significant increases in LPS-specific IgG_1, IgG_{2a}, and IgG_{2b} were observed when compared to O-PS alone.

EFFECT ON CELL-MEDIATED IMMUNE RESPONSES

One unique and important immune modulation property of the QS-21 adjuvant is its ability to induce antigen-specific, class I major histocompatibility complex (MHC) antigen-restricted, CD8+ cytotoxic T lymphocytes (CTLs) when used in viral subunit vaccine formulations. This property was investigated using soluble OVA or OVA adsorbed onto aluminum hydroxide as immunogens (8). C57BL/6 mice that were immunized with soluble native or denatured OVA in formulations that contained increasing quantities of QS-21 raised CTL responses that were specific for the immunodominant epitope of OVA ($OVA_{258-276}$). Similar responses were induced using aluminum hydroxide-adsorbed OVA when mixed with the QS-21 adjuvant, but not when the aluminum hydroxide–adsorbed OVA was used alone (Figure 1). The CTL activity was totally destroyed by treatment in vitro with monoclonal antibody specific to the CD8 antigen plus complement.

Comparison testing of experimental HIV-1 vaccines containing aluminum hydroxide–adsorbed recombinant HIV-1 gp160 protein and the QS-21 adjuvant to formulations containing only the aluminum hydroxide–adsorbed gp160 has been conducted using BALB/c mice and rhesus macaques. Cell-mediated immune responses were increased similarly, and this included the recognition of additional epitopes. Class I MHC antigen-restricted CTLs that were specific for the V3 loop were also raised but, again, only if the QS-21 adjuvant was part of the formulation. Testing using rhesus macaques has demonstrated that the QS-21 is functionally active and safe for use in primates (20).

STRUCTURE OF QUILLAJA SAPONINS

A full understanding of the mechanism of action of these purified saponins requires an understanding of their structures. Studies on alkaline hydrolysis products isolated from

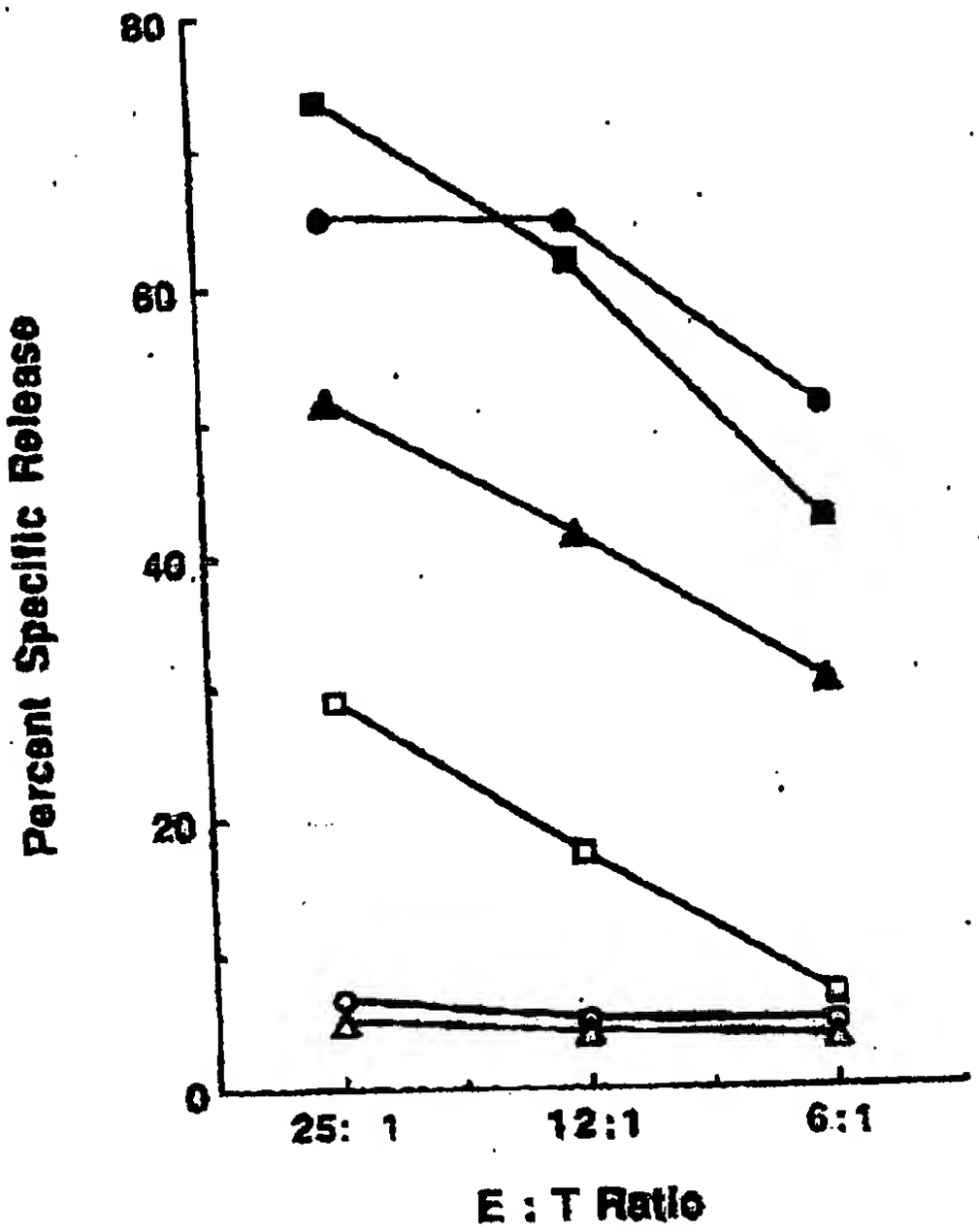

Figure 1 CTL activity measured using EL4 cells expressing OVA as target cells [E.G7-OVA cells (19)] and splenic mononuclear cells obtained from C57BL/6 mice following three immunizations with native soluble OVA (o), denatured soluble OVA (Δ), or OVA adsorbed to alum (□) and the same formulations mixed with 20 μg/dose of QS-21 (shown as solid symbols). Maturation of precursor CTL to functional effector cells in vitro was effected using irradiated E.G7-OVA cells (8). Responses are shown as "percent specific lysis" using a titration of effector to targets (E:T ratio).

crude extracts of *Q. saponaria* showed that the predominant components were quillaic acid 3,28-O-bisglycosides (21,22). An intact saponin, designated QS-III, was isolated and determined to be a quillaic acid 3,28-O-bisglycoside with a fatty acid in ester linkage to the 3 position of fucose [linked to C_{28} of the triterpene (23)]. Determination of the monosaccharide compositions of QS-7, -17, -18, and -21 (7,9) indicated that these purified saponin adjuvants were closely structurally related to each other and to QS-III. Fast atom bombardment—mass spectroscopy indicated that the molecular weights of QS-17, QS-18, and QS-21 were 2321, 2174, and 2012, respectively, with the differences in molecular weight corresponding to an additional glucose in QS-18 compared to QS-21 and an additional rhamnose in QS-17 compared to QS-18. Figure 2 shows the proposed structures of QS-17, QS-18, and QS-21, derived from comparison of our monosaccharide composition and molecular weight

Quillaja saponaria Molina

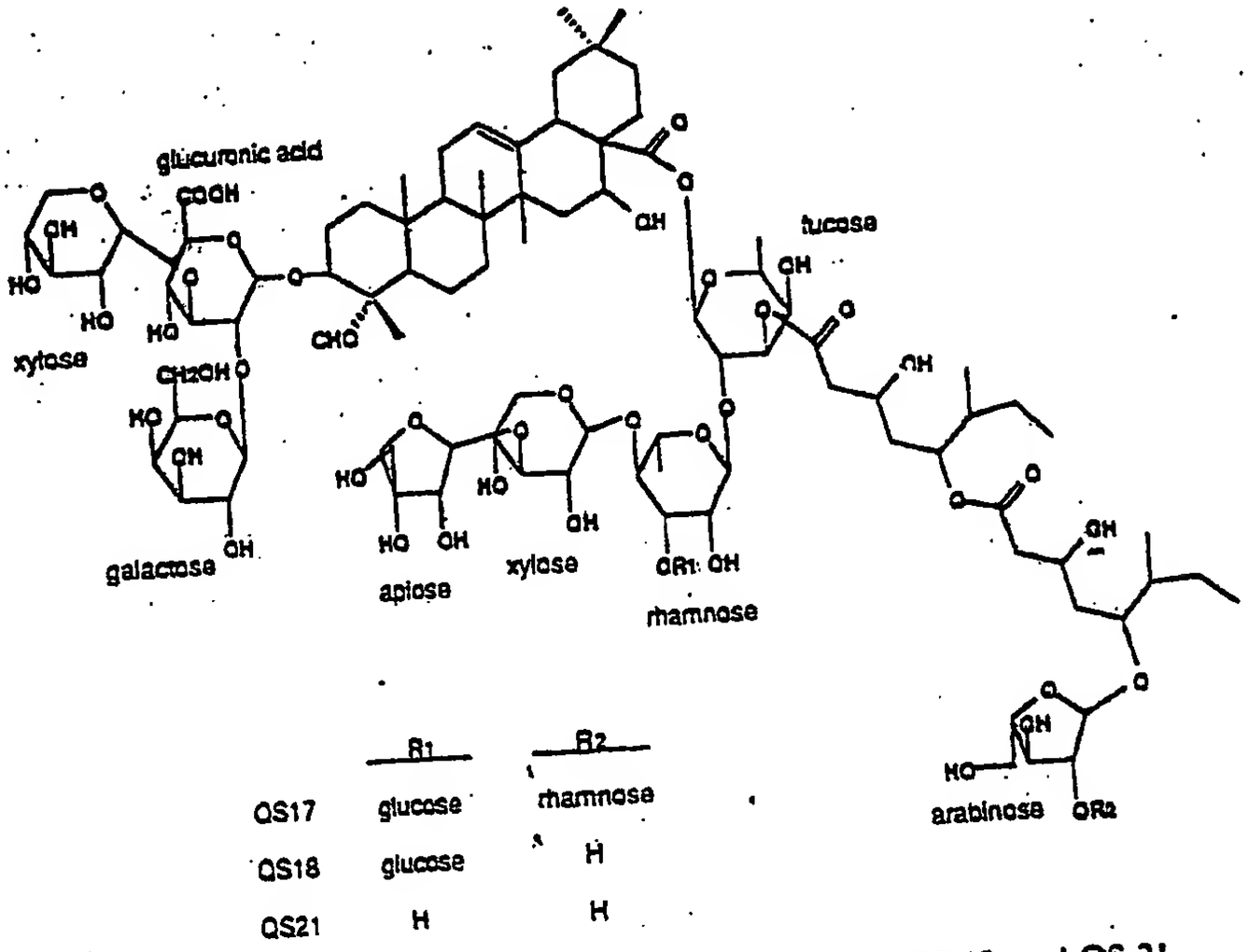

Figure 2 Proposed structure and relationship of QS-17, QS-18, and QS-21.

data for these purified saponins with those of the closely related saponin QS-III that was isolated and characterized by Higuchi. The structure of QS-7 is still unknown, although monosaccharide analysis indicates that its glycoside composition is related to that of the other major saponin adjuvants.

Several functional groups on the saponins have been shown to be important for immune response. Deletion of the fatty acid from either QS-18 or QS-21 substantially reduced the stimulation of antibody titer by the resulting truncated triterpene glycoside compared to the intact saponins [evaluated in CD-1 mice with the antigen BSA (9)]. Periodate oxidation of QS-18 (containing two periodate-sensitive monosaccharide residues, galactose and apiose) destroyed the capacity of this saponin to stimulate antibody titers to BSA, indicating that one or both of these residues are critical to the adjuvant activity of this saponin (9). Studies are under way to identify other critical sites on the saponin adjuvants. It is possible that separate sites on these molecules are responsible for the antibody stimulation and the CTL-stimulation activities.

FORMULATIONS

The vaccine formulations of QS-21 and other purified saponins from *Q. saponaria* described in this review were soluble aqueous preparations prepared by simple admixture of saline solutions of purified saponin and antigen and were not prepared as

emulsions or ISCOMs. The saponins were shown to be effective as adjuvants at concentrations that are below the estimated critical micellar concentration (approximately 100 μg/ml for QS-21 in phosphate-buffered saline pH 7.2), indicating that the micellar structure of the saponins is not critical to the adjuvant response.

We have also demonstrated that QS-21 can be successfully mixed into vaccine formulations that are already adjuvanted with aluminum hydroxide. QS-21 (at a dose of 10 μg) boosted total IgG titers by a factor of 10-fold when added to recombinant FeLV gp70 antigen absorbed with aluminum hydroxide (24) after a single intradermal immunization. Isotype switching from predominantly IgG_1 with aluminum hydroxide alone to IgG_1, IgG_{2b}, and IgG_{2a} in the QS21/aluminum hydroxide formulation was also observed with this antigen (25). This effect has also been observed with aluminum hydroxide–absorbed recombinant HIV envelope antigen (16), with total antigen-specific IgG titer increases of 25–125-fold in BALB/c mice after two immunizations with recombinant HIV-1 gp160 absorbed to aluminum hydroxide and 10 μg QS-21 compared to immunization with the aluminum hydroxide–absorbed protein only. Hence, the saponins may be utilized successfully in fully soluble vaccine formulations as well as in formulations adjuvanted with a solid-matrix adjuvant such as aluminum hydroxide.

We have previously suggested that a close association between the saponin adjuvant and the antigen is important for optimal immune response (7). Such association may occur through hydrophobic interaction between the amphipathic adjuvant and hydrophobic crevices on the protein antigen. Although we have seen clear adjuvant effects with antigens that do not bind QS-21, the adjuvant effect is higher when the antigen is denatured, allowing an increased binding of QS-21 (data not shown). Hence, we expect that a forced association of antigen and adjuvant through covalent linkage may improve immune response to small, hydrophilic proteins or peptides that ordinarily do not bind QS-21.

Similar approaches to vaccine formulation have been utilized successfully with the adjuvant muramyl dipeptide (MDP) (26,27). To investigate this type of formulation with the purified saponin adjuvants, we covalently coupled QS-21 to hen-egg lysozyme (9). This conjugation was carried out using carbodiimide chemistry to directly couple the QS-21 glucuronic acid to protein amino groups. A 1:1 molar conjugate was tested in C57BL/6 mice and compared to the same ratio of unconjugated lysozyme and QS-21. No antibody response was observed in these mice with the unconjugated lysozyme, consistent with this being a nonresponder strain to lysozyme. However, an antibody response was induced by the 1:1 molar conjugate. Still higher responses were induced when additional free QS-21 was added to the conjugate, suggesting that the conjugated QS-21 provided a binding site for additional QS-21 to bind to lysozyme, further enhancing the antibody stimulation. Hence, although with most antigens the purified saponin adjuvants will boost immune responses in unconjugated forms, vaccine formulations containing conjugated antigen and adjuvant show promise for boosting response to antigens that are otherwise not well-adjuvanted by the

saponins. We are currently testing immune responses to conjugates prepared from peptides and QS-21 to determine whether such formulations will be useful in vaccines against HIV-1.

TOXICITY OF DIFFERENT PURIFIED SAPONINS

One of the drawbacks of utilizing crude or partially purified saponins as an adjuvant is the toxicity associated with these preparations, which has been noted by several investigators (3,5). However, in a test of HPLC-purified saponins, it was found that the adjuvant active saponins covered a wide range of toxicity (assessed by lethality in mice); the saponin QS-18 was considerably more toxic than the original bark extract and the saponins QS-7 and QS-21 were considerably less toxic (7). No correlation was made between adjuvant activity and toxicity because both toxic and nontoxic saponins were adjuvant-active in a similar dose range in mice. In addition, no correlation was made between the hemolytic activity of the saponins and toxicity in mice (7).

It is important to note that the saponin QS-18, which is the most toxic component in mice, is the predominant saponin in all samples of *Q. saponaria* bark and commercial saponin samples that we have analyzed, suggesting that it may be responsible for much of the toxicity in mice that has been noted with these samples. Our approach to the use of saponins as adjuvants in vaccines is to use homogeneous saponin components with well-defined adjuvant potency and toxicity rather than partially purified extracts that may vary in proportions of toxic and adjuvant-active components. The ideal adjuvant candidate would be a saponin that is more potent as an adjuvant than the original extract but considerably less toxic. QS-21 shows considerable potential as such an adjuvant. Tests of QS-21 doses of 50 μg administered to rabbits by the intramuscular route showed that there was no observable effect on clinical hematology and serum chemistry data or on gross and microscopic pathology, after administration of four doses over a 2-week period (data not shown). Field tests of a commercial FeLV vaccine containing QS-21 as adjuvant showed that this vaccine was safe for use in cats (17); in addition, an experimental HIV-1 vaccine containing 50 μg QS-21 was used safely in primates (20).

SUMMARY

Individual saponins from *Quillaja saponaria* can be purified to homogeneity, allowing an immunological and structural characterization of the predominant adjuvant components from this species. One of these saponins, QS-21, has been tested extensively as an adjuvant in mice. It shows potent activity for stimulation of antigen-specific antibody titers to T-dependent antigens, including significant increases in IgG_{2a} and IgG_{2b} as well as in IgG_1, augments class I MHC-restricted CTLs to subunit vaccines and, surprisingly, augments an increase in antibody titer to T-independent antigens.

The cellular mechanism by which QS21 evokes these responses is still unclear. Future studies will be directed to elucidation of this mechanism as well as of the sites on the QS-21 molecule required for the adjuvant responses.

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